The development of new inoculation techniques and viability tests for *Neotyphodium* endophytes

A thesis submitted in partial fulfilment of the requirements for the Degree

of

Master of Science in Plant Biotechnology

at the

University of Canterbury

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University of Canterbury

2007

Abstract

Neotyphodium endophytes (Claviceptaceae) are asexual filamentous fungi found living between the cells of many cool season forage grasses including tall fescue, meadow fescue and perennial ryegrass. They produce a range of alkaloids, including ergovaline and lolitrem B, which have been shown to be directly associated with the livestock disorders fescue toxicosis and ryegrass staggers syndrome, while others, including peramine and the lolines, have been linked to increased insect and drought resistance of the grass host. In the past decade, the *Neotyphodium* strains AR1, MaxQ and MaxP were selected because they did not produce the alkaloids associated with livestock disorders. Subsequently, artificial associations were established between them and commercial forage grass cultivars.

The slow growth rate of *Neotyphodium* endophytes *in vitro* and the low success rate of the present methods for establishing artificial associations between endophytes and grass hosts are limiting the rate at which new novel endophytes can be incorporated into plant breeding programs and eventually commercialised. In this thesis, the type and concentration of the growth medium was shown to affect radial growth rate, colony appearance and mycelial morphology of three strains of *Neotyphodium* endophytes. The floret inoculation of meadow fescue with the U2 strain of *N. uncinatum* using several techniques involving liquid culture was attempted but was unsuccessful in creating any artificial associations.

Neotyphodium endophytes are unstable in stored seed. In New Zealand, it is critical that pastures are infected with protective *Neotyphodium* endophytes to ensure that they will not be destroyed by exotic pests. The present methods for determining the percentage of viable endophyte infection of a seed lot are too slow for efficient use in the commercial seed industry. In this thesis, primers specific to the β-tubulin gene of *N. coenophialum*, *N. lolii* and *N. uncinatum* were designed and successfully used to detect these species *in planta*. However, using these primers to develop a method to accurately determine the viable endophyte infection rate of a seed lot using RT-qPCR was unsuccessful.

Acknowledgements

I would like to acknowledge Cropmark Seeds Ltd. for supplying the majority of the funding for this thesis and the employment contract that was offered to, and accepted by me upon its completion.

Personally I would like to acknowledge:

My supervisors, Professor Paula Jameson and Dr Jason Song (School of Biological Sciences, University of Canterbury, Christchurch), for their help in planning, advising me throughout, and editing this thesis.

Nick Cameron (Cropmark Seeds Ltd., Darfield) for the supply of plant and fungal materials.

Dr Ian Harvey (Plantwise, Lincoln) for his advice on the *in vitro* culture of *Neotyphodium* endophytes.

Nick Grbavac (Agriquality, Lincoln) for the isolation and supply of common contaminant fungal species found on commercial forage grasses.

My parents, Andrew and Glenys Gillanders for supporting me financially throughout university.

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Chapter One

Introduction

1.1. Background

Agricultural exports make up 50-60% of New Zealand's annual export earnings and contribute 20 percent to the gross domestic product of the country (www.maf.govt.nz). Many of these exports, such as dairy, meat, and wool products, are derived from livestock which feed predominantly on pasture. Unique climatic conditions allow New Zealand farmers to feed their stock almost entirely on fresh pasture the whole year round, and the majority of supplementary feeds (hay and silage) are produced from cut pasture (Woodfield and Easton, 2004). Consequently, the pastoral industry, and research into producing more desirable grass cultivars, is of considerable importance to New Zealand's economy. Not surprisingly, New Zealand leads the world in many areas of pastoral research (Woodfield and Easton, 2004).

Traditional plant breeding techniques have been used in the pastoral industry for decades to produce more and more desirable grass cultivars (Woodfield and Easton, 2004). However, these techniques are beginning to reach their limits as pair-wise crossing of two plants with desirable characteristics will produce a plant that it is only slightly more desirable than its parents (pers. comm., Nick Cameron, Cropmark Seeds Ltd). Plant breeders now believe that the most desirable grass cultivar will be a composite cross containing desirable characteristics from the four main forage grass species *Lolium perenne* (perennial ryegrass), *Festuca arundinacea* (tall fescue), *Festuca pratensis* (meadow fescue) and *Lolium multiflorum* (Italian/annual ryegrass) (Thomas and Humphreys, 1991)(pers. comm., Nick Cameron, Cropmark Seeds Ltd.). Alteration of the ploidy of these species is required to produce such a cross; this is done using colchicine treatment, to double, and anther culture, to half, the chromosome number of the plant (Ahloowal, 1967; Olesen et al., 1988).

In recent years a group of fungi collectively known as 'endophytes', has been the focus of much research and the fungi have been shown to increase the performance of their grass host (Latch et al., 1985; Arachevaleta et al., 1989; Malinowski et al., 1997). This genus of fungi was named *Neotyphodium* (Glenn et al., 1996). Three species of this genus, *N. lolii* (Latch et al., 1984), *N. coenophialum* (Morganjones and Gams, 1982), and *N. uncinatum* (Gams et al., 1990), and the tritrophic interaction between them, their grass hosts (*L. perenne*, *F. arundinacea*, *F. pratensis* respectively), and the livestock that feed on the grass, have been at the centre of this research (Woodfield and Easton, 2004) (Schardl et al., 2004).

Cropmark Seeds Ltd. (the company supplying the majority of funding for the research reported in this thesis), hold the patent to a strain of *N. uncinatum* called 'U2'. In the near future Cropmark Seeds Ltd. plans to commercialise this strain by establishing artificial associations between it and some of their best performing grass cultivars. This research was undertaken to increase the understanding of *Neotyphodium* endophytes, with a particular focus on the U2 strain of *N. uncinatum*, and to try and develop new methodology that could be used in endophyte science.

1.2. The *Neotyphodium* endophytes

For many years grasses have been known to form relationships with a group of fungi collectively known as 'endophytes' (Freeman, 1904; Sampson, 1937, 1939; Neill, 1941). In some grasses the intercellular mycelia of these endophytes may be present throughout the plant while, in others, mycelia are restricted to leaves, culms, and seeds. *Epichloë typhinia* is endophytic to several grass species for most of the year but, just prior to flowering, the mycelia emerge and form a stroma which inhibits or restricts flowering (Sampson and Western, 1954). Apart from the 'choke' type symptoms of *E. typhinia* on some grasses it was generally assumed that endophytes had little or no effect on their host. For this reason little research was carried out on these endophytes up until the early 1980's.

In 1977 a connection was made between endophyte-infected tall fescue pastures and fescue toxicosis in cattle (Bacon et al., 1977). Soon after this, the same connection was observed between endophyte infected perennial ryegrass pastures and ryegrass staggers in sheep (Fletcher and Harvey, 1981). It was then recognised that some of the endophytes that were previously thought to be harmless to the health of grazing animals, were actually responsible for some of the biggest livestock health problems stock farmers were facing (Gallagher et al., 1984; Yates et al., 1985). Soon after this, studies also showed that grass plants infected with endophyte had a higher resistance to attack from some major pest insects (Funk et al., 1983; Latch et al., 1985; Rowan and Gaynor, 1986) and produced a greater amount of dry matter than non-infected plants (Latch et al., 1985). These discoveries, that demonstrated that there was a relationship between endophytes, their grass hosts, and the livestock feeding on the host, led to a significant increase in interest in endophytes from the agricultural and scientific communities.

1.2.1. The phylogeny of *Neotyphodium* endophytes

Endophytes of the grass family *Poaceae* were originally classified into section *Albolanosa* of the genus *Acremonium* (Morganjones and Gams, 1982) in the fungal family *Claviceptaceae* (*Epichloë*), to distinguish them from the generally saprophytic species of sections *Acremonium*, *Chaetomioides*, *Gliomastix*, and *Nectrioidea*. However, taxonomic studies in the 1980's and early 90's involving microscopy (Christensen et al., 1991) (Christensen et al., 1993), alkaloid profiling (Christensen et al., 1991), isozyme analysis (Christensen et al., 1993), and molecular phylogenetic analysis of the beta-tubulin (*tub2*) (Tsai et al., 1994) and the ribosomal internal transcribed spacer (*its*) (Glenn et al., 1996) genes of these and other species of the *Acremonium* family uncovered many problems with this classification. Eventually, in 1996, the genus *Neotyphodium* (Glenn et al., 1996) was erected. This genus included all the conidial fungi that had previously been classified into *Acremonium* section *Albolanosa*. Phylogenetic studies indicated that *Neotyphodium* spp. evolved from *Epichloë* spp., with most being interspecific hybrids with close affinities to two or more *Epichloë* spp. *N. coenophialum* (Morganjones and Gams, 1982), native to *Festuca arundinacea* (tall fescue), *N. lolii* (Latch et al., 1984),

native to *Lolium perenne* (perennial ryegrass) and *N. uncinatum* (Gams et al., 1990) native to *Festuca pratensis* (meadow fescue) became the focus of most research due to their respective hosts being economically important forage grasses.

1.2.2. The growth and life cycle of *Neotyphodium* endophytes *in planta*

Within the grass plant the endophyte follows very distinct basal to apical hyphal concentration gradients (Herd et al., 1997; Christensen et al., 1998) being more abundant in leaf sheaths than blades (Christensen et al., 1997). The hyphae appear to originate from profusely branching mycelium in the basal meristems (Christensen et al., 2002) of the grass plant. In the expanding and mature leaf tissues, the hyphae are rarely branched and are arranged mainly along the longitudinal axis of adjacent plant cells (Fig. 1.1) (Christensen et al., 2002). The hyphae are in close contact with the plant cells and appear to be firmly attached, but they never breach host cell walls nor develop conspicuous feeding structures such as haustoria or arbuscules (Christensen et al., 2002; Schardl et al., 2004). What causes the transition from highly branched hyphae in the plant base to single, thread-like hyphae in the leaf sheaths is unknown, but may involve physical restrictions to branching as well as biochemical cues released from the surrounding plant cells (Schardl et al., 2004). Within the plant, meristems represent strong sinks providing a nutrient rich environment for hyphal growth (Schardl et al., 2004). In such an environment the endophyte may not need to alter or damage the host cells to obtain the necessary nutrients for profuse growth (Schardl et al., 2004). However, it would seem that vascular bundles would also represent a nutrient sink. In native (natural) associations hyphae are seldom present in vascular bundles although highly colonised vascular bundles can be present in the leaves of some artificial (inoculated) associations (where the strain of endophyte is not native to its host) (Christensen et al., 1997). Within leaves they are most abundant amongst the mesophyll cells (Christensen et al., 2002). Hyphae have never been observed growing intracellularly or in root tissue.

The growth of *Neotyphodium* endophytes in host grasses is highly regulated (Christensen et al., 2002). Hyphae grow within developing leaves and cease apical extension and

branching when leaf growth is complete (Herd et al., 1997; Schmid et al., 2000; Tan et al., 2001). Several enzymes putatively involved in nutrient acquisition have been recently discovered (Lyons et al., 1990; Lindstrom et al., 1993; Lam et al., 1994, 1995; Reddy et al., 1996; Moy et al., 2002), though some of these could also be involved in hyphal growth and branching, and perhaps the suppression of host defenses.

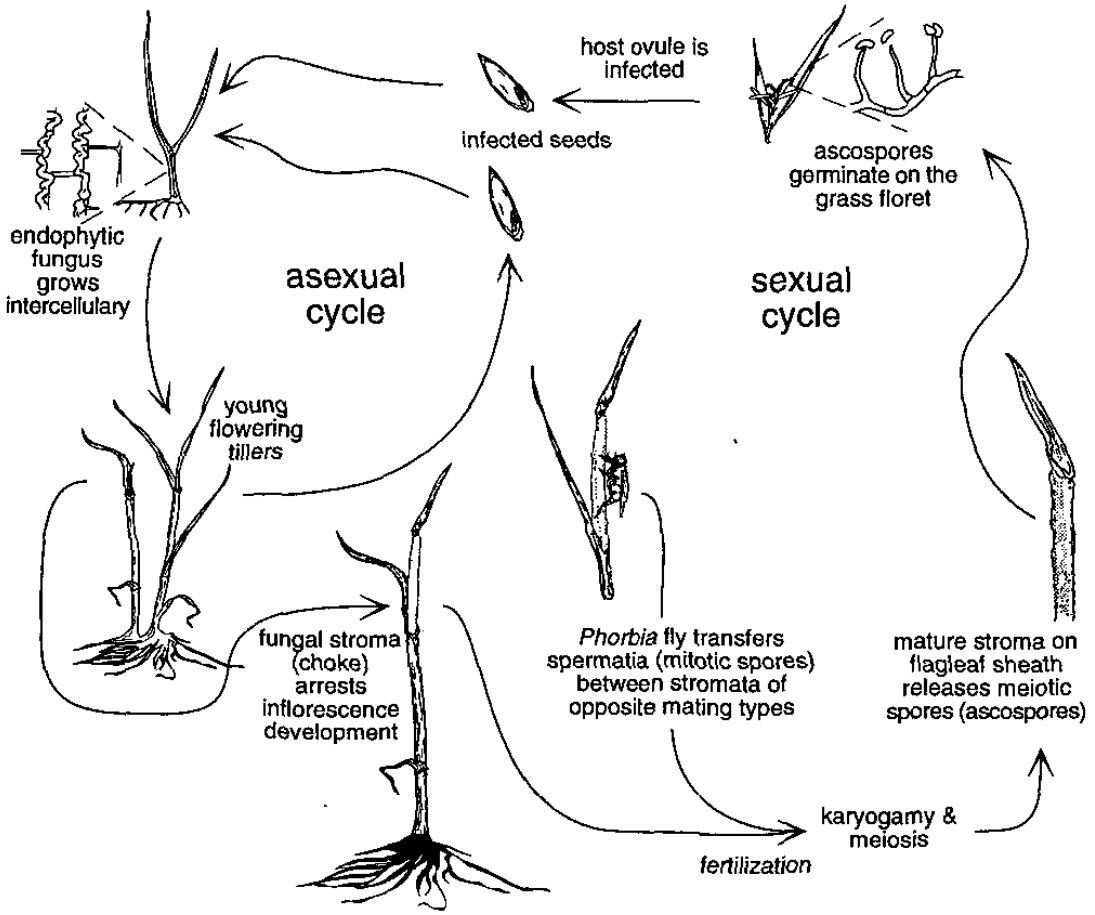


Figure 1.1. The sexual and asexual life cycles of *Epichloë* and *Neotyphodium* endophytes (Bush et al., 1997). *Neotyphodium* endophytes reproduce strictly via the asexual cycle.

In mutualistic associations, where one organism is contained within another organism, selection against sexual reproduction of the endosymbiont frequently occurs (White, 1988). Years of asexual reproduction often results in the loss of the capacity for sexual reproduction. This phenomenon appears to be evident amongst the *Neotyphodium* endophytes (White, 1988). Whereas their *Epichloë* relatives are often able to reproduce sexually (through sporulation of stroma on the epidermis of the host) and asexually

(through vertical transmission) (Fig. 1.1), *Neotyphodium* endophytes reproduce strictly asexually. The hyphae simply grow up the seed head, into floral meristems, and then into the ovules of the florets, infecting the next generation of seed with endophyte (Sugawara et al., 2004). Spermatangia play an integral role in the sexual cycle (Fig. 1.1). Without the continued use of spermatangia, White (1988) suggests that mutations may accumulate affecting the capacity of the endophyte to produce them (White, 1988). *N. lolii* and *N. uncinatum* appear to be in this process (White, 1988) as evidenced by the slow growth and abnormal development of conidiogenous cells in culture (Latch et al., 1984; Gams et al., 1990; Christensen et al., 2002). Interestingly, *N. coenophialum* shows no tendency toward losing the ability to form spermatangia *in vitro*, even though stromata have never been observed on a grass host infected with it. This may be explained by the fact that its native grass host, tall fescue, only became extensively cultivated in the mid 20th century (White, 1988).

1.2.3. P-endophytes

A second group of systemic fungal endophytes are also found in the same grass species as *Neotyphodium* endophytes (Latch et al., 1984; An et al., 1993; Christensen et al., 2002). These endophytes have not yet been formally described and have been referred to as p-endophytes that is, endophytes with penicilliate conidiophores (An et al., 1993). Their growth contrasts strongly with that of the *Neotyphodium* endophytes appearing to be unregulated and consisting of highly branched hyphae present in higher concentrations in outer older leaf sheaths than in the sheaths of newly formed leaves (Philipson, 1989).

1.3. The production and properties of alkaloids produced by *Neotyphodium* endophytes.

Pest resistance and the adverse effects of *Neotyphodium* endophytes on livestock are ascribed to different alkaloid groups synthesised *in planta* by the endophyte (Lane et al., 2000). The effects the alkaloids have on both vertebrates and invertebrates that feed on the grass host have been widely studied (Johnson et al., 1986; Rowan and Gaynor, 1986; Fletcher and Easton, 1997). In more recent years much research has also gone into elucidating the biochemical pathways that are involved in the synthesis of the different alkaloid groups as well as finding the genes that encode the enzymes that catalyse these pathways (Panaccione et al., 2003; Young et al., 2006). The four major alkaloid groups are the ergopeptines, the lolitrems, the lolines, and peramine (Bush et al., 1997). A fifth group known as janthitrems has recently been discovered, but only one strain of *N.lolii* is as yet known to produce them (Tapper and Lane, 2004). Most endophytes produce a subset of these four classes (Table 1.1) (Bush et al., 1997).

Table 1.1. The subsets of the different alkaloid groups produced by the three major *Neotyphodium* species

Endophyte species	Lolitrem B	Ergovaline	Peramine	Lolines
N. coenophialum	No	Yes	Yes	Yes
N. lolii	Yes	Yes	Yes	No
N. uncinatum	No	Yes	Yes	Yes

This does not mean to say that all the different strains of a species produce the subset of alkaloids stated in Table 1.1. Different strains vary both quantitatively and qualitatively in their ability to produce the different alkaloids groups. The AR1 strain of *N. lolii* lacks the ability to produce lolitrem B and ergovaline and only produces peramine (Fletcher, 1999). The U2 strain of *N. uncinatum* only produces peramine and the loline alkaloids (Cropmark Seeds Ltd., unpublished data). The MaxP strain of *N. coenophialum* produces a lot less ergovaline than wild type strains of its species (Bouton and Easton, 2004).

Endophyte strains with these unique alkaloid profiles, differing from that of the wild type strains of their species, are often referred to as 'novel' strains.

The amount of each alkaloid group produced also varies throughout the year (Belesky et al., 1988; Ball et al., 1995) and in response to the environment (Belesky et al., 1988; Lane et al., 1997b). It has also been shown to vary between individual grass hosts even if they are grown in the same conditions (Latch, 1994; Ball et al., 1995; Easton et al., 2002). In addition to these alkaloid groups, it has become clear that a diversity of bioactive metabolites are also produced by different endophyte strains (Lane et al., 2000; Popay and Bonos, 2004).

1.3.1. The indole-diterpene lolitrem B

Indole-diterpenes are a large structurally diverse group of natural products principally found in filamentous fungi of the genera *Penicillium*, *Aspergillus*, *Claviceps*, and *Neotyphodium* (Young et al., 2006). Many of these compounds are potent tremorgenic mycotoxins. The most abundant indole-diterpene found in the *N. lolii*-perennial ryegrass association endophytes is lolitem B (Gallagher et al., 1984). Lolitrem B is not synthesised by either *N. coenophialum* or *N. uncinatum*. Whether these species have the biosynthetic pathway for producing lolitrem B or the pathway is blocked at some point is unknown.

The link between lolitrem B and the mammalian mycotoxin disorder, 'ryegrass staggers syndrome', has encouraged a systematic analysis of the indole-diterpene profile in *N.lolii*-infected perennial ryegrass seed and led to a proposed metabolic grid for the biosynthesis of lolitrem B and related indole-diterpenes (Fig. 1.2) (Munday-Finch et al., 1998; Gatenby et al., 1999; Young et al., 2005; Young et al., 2006).

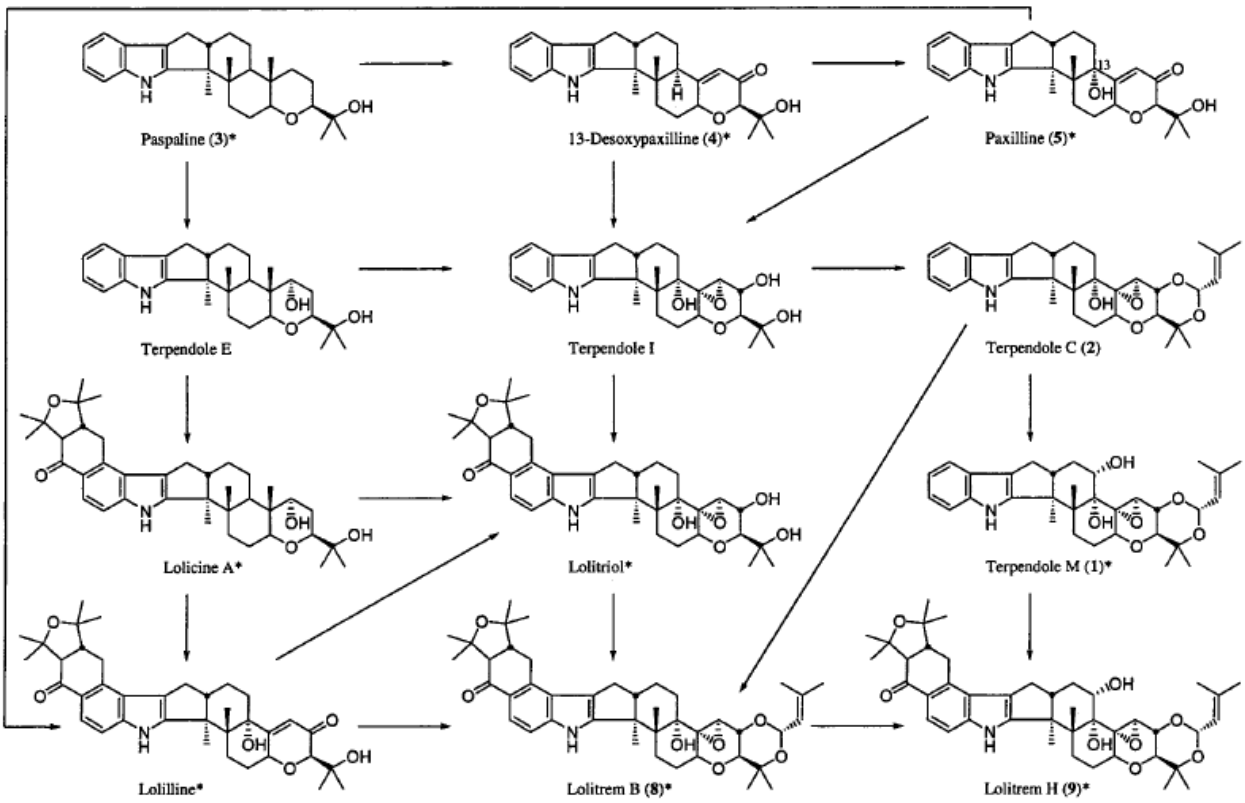


Figure 1.2. Possible metabolic grid for the biosynthesis of lolitrem B involving known paspaline, paxilline, and terpendole analogues. Compounds marked with an asterisk have been isolated from endophyte infected *L. perenne* (Gatenby et al., 1999).

The structural similarity between the diverse array of indole-diterpenes identified in *N. lolii*-infected perennial ryegrass and paxilline, an abundant metabolite of *Penicillium paxilli*, suggested that these compounds are derived from either paxilline or proximate precursors of paxilline, such as paspaline (Young et al., 2006). Using paxilline biosynthesis in *P. paxilli* as a model, Young et al. (2006) identified a complex genetic locus of at least three lolitrem (*ltm*) gene clusters which control indole-diterpene biosynthesis in *N. lolii* (Young et al., 2006).

1.3.2. The ergopeptine ergovaline

Ergopeptines are produced by several species of phytopathogenic and endophytic fungi, most notably species of the genus *Claviceps*, common in grain crops. With regard to the

production of them by *Neotyphodium* endophytes, Yates et al. (1985) first conclusively identified this class of alkaloids in tall fescue seed infected with *N. coenophialum* (Yates et al., 1985). Ergovaline is the most predominant ergopeptine alkaloid produced by *Neotyphodium* endophytes, along with lesser amounts of ergosine, ergotamine, and other lysergic acid derivatives (Yates et al., 1985; Lane et al., 2000). As is the case with *Claviceps purpurea* (ergot) infestation of grains crops, these alkaloids in tall fescue have been shown to produce a spectrum of detrimental pharmacological effects in animals consuming *N. coenophialum*-infected forage, commonly known as fescue toxicosis. These effects include, but are not limited to, poor weight gain, low milk production, poor reproduction, depressed immune response, gangrene of lower limbs, and various direct endocrine effects (Bacon, 1995; Cross et al., 1995; Porter, 1995). In the case of *N. lolii*, which produces ergovaline along with lolitrem B, ergovaline has been shown to exacerbate the effects of lolitrem B by increasing the intensity of ryegrass staggers (Fletcher and Easton, 1997). *N. uncinatum* typically produces a lot less ergovaline than *N. coenophialum* and *N. lolii* (Cropmark Seeds Ltd, unpublished data). The production of ergovaline by *N. lolii* has also been shown to deter Black beetle (*Heteronychus arator*) from feeding on its host, perennial ryegrass, and may also deter other insects (Ball et al., 1997).

Ergopeptines are non-ribosomally synthesised peptides containing lysergic acid and three amino acids that vary between, and define, the molecules in the family (Panaccione et al., 2001). Ergovaline contains D-lysergic acid, L-alanine, L-valine, and L-proline, and differs from ergotamine, the most common ergopeptine produced by the ergot fungus *Claviceps purpurea*, in containing L-valine in place of L-phenylalanine (Brunner 1979). The assembly of ergopeptines (Fig. 1.3) is catalysed by a multifunctional peptide synthetase complex named lysergyl peptide synthetase (Riederer et al., 1996; Walzel et al., 1997). Unlike other eukaryotic peptide synthetases in which all activities are encoded on a single polypeptide, lysergyl peptide synthetase is made up of two separate polypeptides (Panaccione et al., 2001). Lysergyl peptide synthetase 2 (LPS2) activates lysergic acid by adenylation and then binds it covalently as a thioester to enzyme-bound 4`-phosphopantethenate, prior to its transfer to lysergyl peptide synthetase 1 (LPS1).

LPS1 recognises the three amino acids of the peptide portion of the ergopeptine, binds them as thioesters via adenylate intermediates, and then assembles the lysergyl peptide. The product of this multifunctional enzyme complex is lysergyl peptide lactam. The lactam is then oxidised at the α-carbon of the first amino acid in the peptide chain. This hydroxylated intermediate is thought to spontaneously cyclise to the final cyclol ergopeptine product (Panaccione et al., 2003).

Genes encoding LPS1 have been cloned from *C. purpurea* and *Neotyphodium* sp. Lp1 (Panaccione et al., 2001). The genes contain three characteristic peptide synthetase modules, consistent with the role of this enzyme in attaching three amino acids to lysergic acid. Panaccione et al. (2001) made a targeted gene knockout in the LPS1 encoding gene *lpsA* in the perennial ryegrass endophyte *Neotyphodium* sp. Lp1 (Fig. 1.3) (Panaccione et al., 2001). Analysis of perennial ryegrass infected with this knockout mutant showed that ergovaline was no longer produced (Panaccione et al., 2001). The amount of lysergic acid that accumulated in the *lpsA* knockout associations corresponded to only thirteen percent of the concentration of lysergic acid derivatives that accumulated in Lp1 associations, indicating altered regulation of the pathway via a feedback mechanism (Panaccione et al., 2003).

The first step in the ergovaline biosynthetic pathway has also been successfully disrupted, blocking the production of ergovaline and any of the lysergyl or clavine precursors (Wang et al., 2004). Dimethylallyltryptophan (DMAT) synthetase is the enzyme that catalyses the formation of DMAT from tryptophan and dimethylallylpyrophosphate (DMAPP) (Fig. 1.3). DMAT synthetase was purified from *Claviceps fusiformis* and characterised as a homodimer of 52 kDa subunits (Gebler et al., 1992). The *dmaW* gene was then identified and cloned from *C. purpurea* (Tsai et al., 1995; Tudzynski et al., 1999). Wang et al. 2004 successfully identified and cloned the *dmaW* gene in *Neotyphodium* sp. Lp1 (Wang et al., 2004). It was then successfully knocked out and the mutant failed to produce any detectable ergovaline or simpler ergot and clavine alkaloids (Wang et al., 2004).

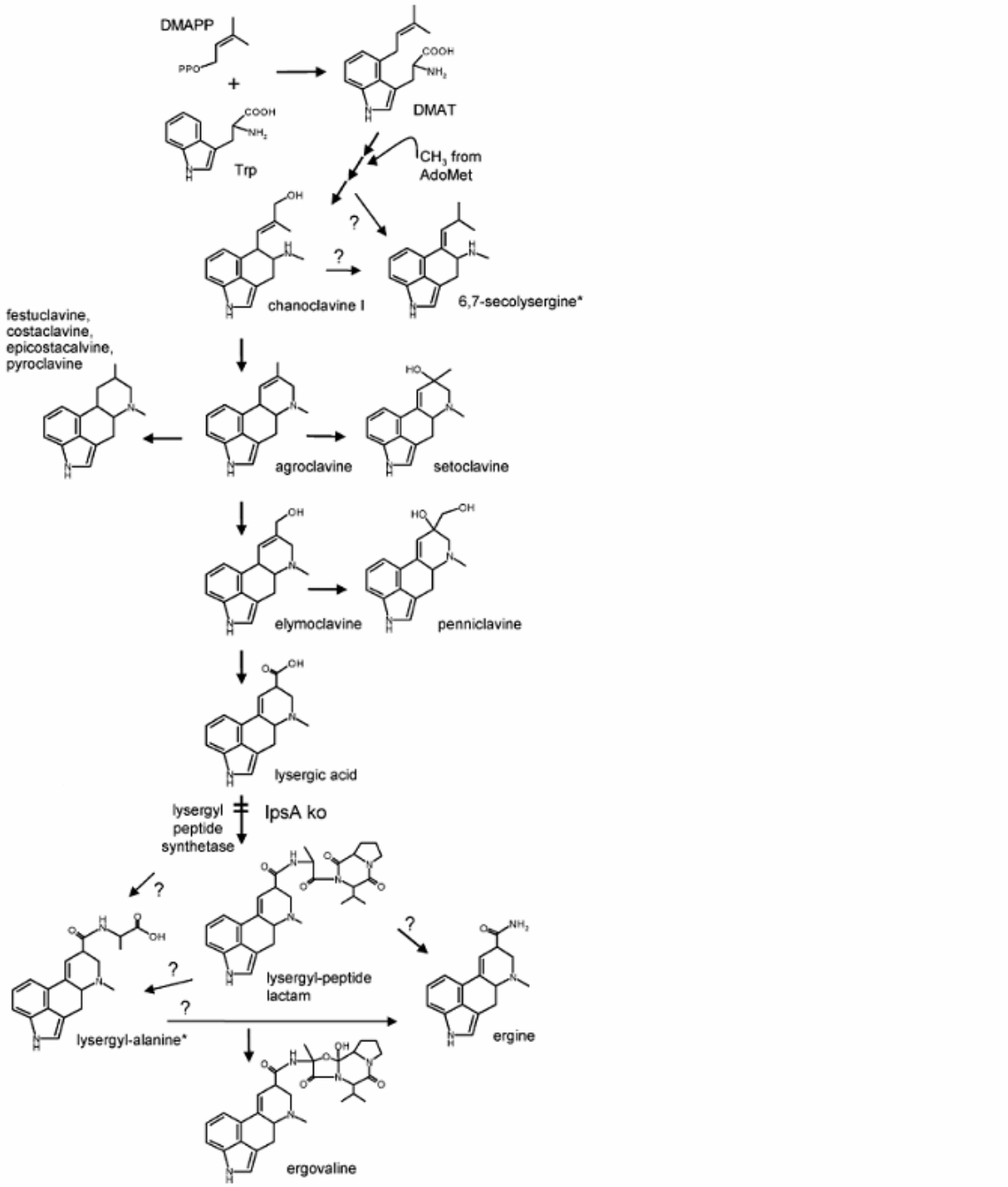


Figure 1.3. The biosynthetic pathway for the production of ergovaline. Stuctures and common names of key intermediates and products of the ergot alkaloid pathway and associated spurs Abbreviations; DMAPP = dimethylallylpyrophosphate; Trp = tryptophan; DMAT = Dimethylallyltryptophan; AdoMet = S-adenosyl-methionine; lpsA ko = step at which the pathway that has been knocked out in that particular mutant. Question marks indicate uncertain relationships between intermediates and/or products. Additional pathway spurs or modifications of listed alkaloids have been omitted in cases where the products were absent or were minor components of the endophyte ergot alkaloid profiles (Panaccione et al., 2003).

1.3.3. Peramine

Peramine, a pyrrolopyrazine alkaloid, actively deters feeding by some species of insects (Rowan and Gaynor, 1986; Prestidge and Gallagher, 1988). One of the most dramatic examples of this involves the Argentine stem weevil (*Listronotus bonariensis*), a devastating pest of perennial ryegrass in New Zealand. If undeterred this exotic pest feeds on the crowns, and, in much of New Zealand, can eradicate entire stands of pasture (Schardl et al., 2004). *N. lolii* effectively protects the grass from the stem weevil through its production of peramine (Rowan and Gaynor, 1986; Prestidge and Gallagher, 1988). For this reason almost all perennial ryegrass sown in New Zealand today is infected with the AR1 strain of *N. lolii*, which produces high amounts of peramine, but no ergovaline or lolitrem B. Peramine production in various endophyte-infected grass species also correlates with activity against the aphid *Schizapus graminus* (Siegel, 1990).

The structure of peramine (Fig. 1.4 a) suggested that it was derived from proline and arginine via a diketopiperazine intermediate (Rowan and Gaynor, 1986; Rowan, 1993). The lipophilic ring system and the hydrophilic guanidinium group are novel structural features not reported in any other insect feeding deterent (Tanaka et al., 2005). Peramine is the product of a reaction catalysed by a two-module non-ribosomal peptide sythetase (NRPS) (Tanaka et al., 2005). Tanaka *et al*. (2005) isolated the gene (*perA*) that encoded for this NRPS and through deletion and complementation analysis proved that it was essential for peramine biosynthesis. The similarity of genes adjacent to *perA* with genes from other organisms allowed for the prediction of their functions and the development of a proposed biosynthetic pathway for peramine in *Neotyphodium* and *Epichloë* endophytes (Tanaka et al., 2005) (Fig 1.4 b).

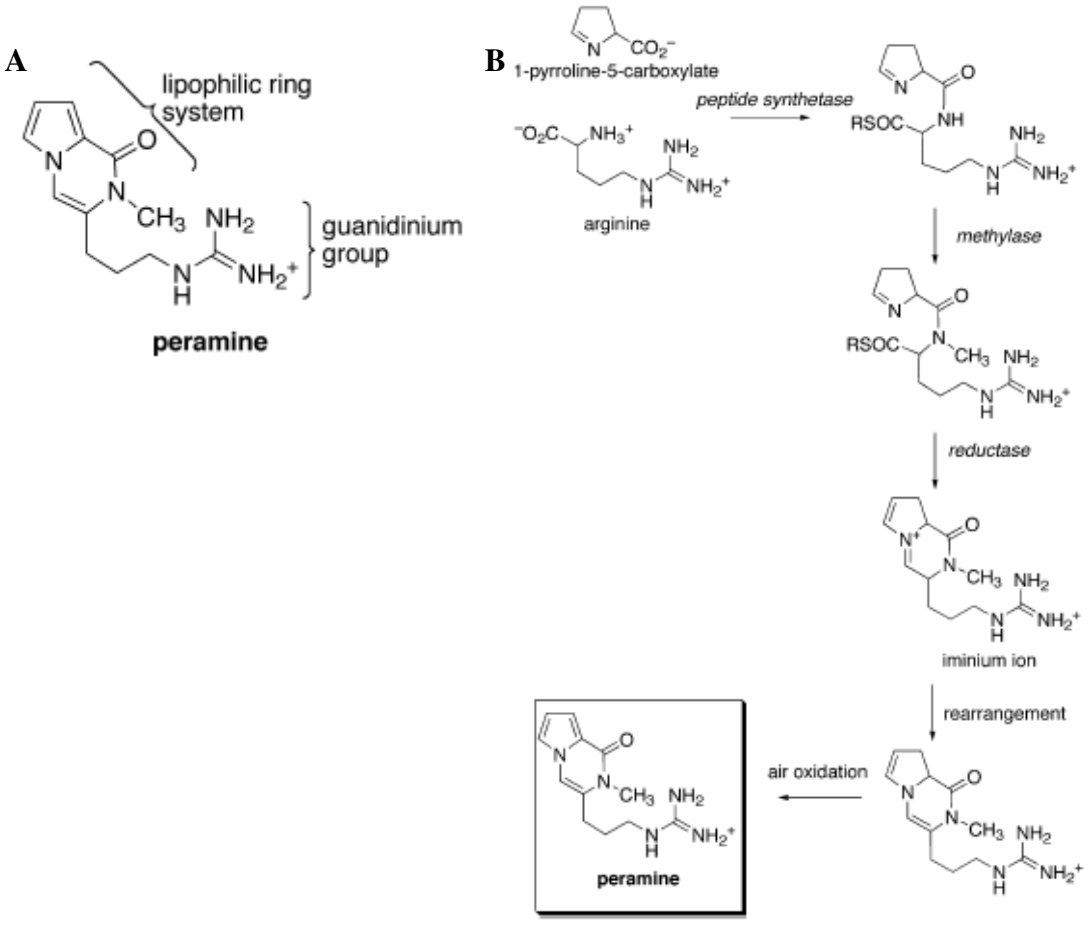


Figure 1.4. The (A) structure and (B) the proposed pathway for the biosynthesis of peramine (Tanaka et al., 2005).

1.3.4. Janthitrems

Early investigations into ryegrass staggers syndrome were directed towards the characterisation of tremorgen-producing *Penicillium* species. Work in the 1980s identified a number of *Penicillium janthinellum* strains isolated from pastures where there had been outbreaks of ryegrass staggers (Gallagher et al., 1980). When these strains were grown in culture tremorgenic compounds named janthitrems were produced. As in the case of lolitrems, janthitrems are derived from tryptophan and have paxilline as a precursor (Penn and Mantle, 1994). Janthitrem B is the most abundantly produced in *P. janthinellum* (Fig. 1.5). However, research into the role of janthitrems in ryegrass

staggers ceased when lolitrems were identified as the probable causative agents. In 2004, a strain of *N. lolii* named AR37 was shown to produce the janthitrem related compound 11, 12-epoxyjanthitrem G (Fig. 1.5) (Tapper and Lane, 2004). AR37 was released commercially in four varieties of perennial ryegrass in 2006 by Grasslanz®. A recent study has shown that epoxy-janthitrems are much less toxic than lolitrem B (Fletcher, 2004). Episodes of staggers recorded in animals grazing AR37 have been infrequent and of short duration (Fletcher, 2004).

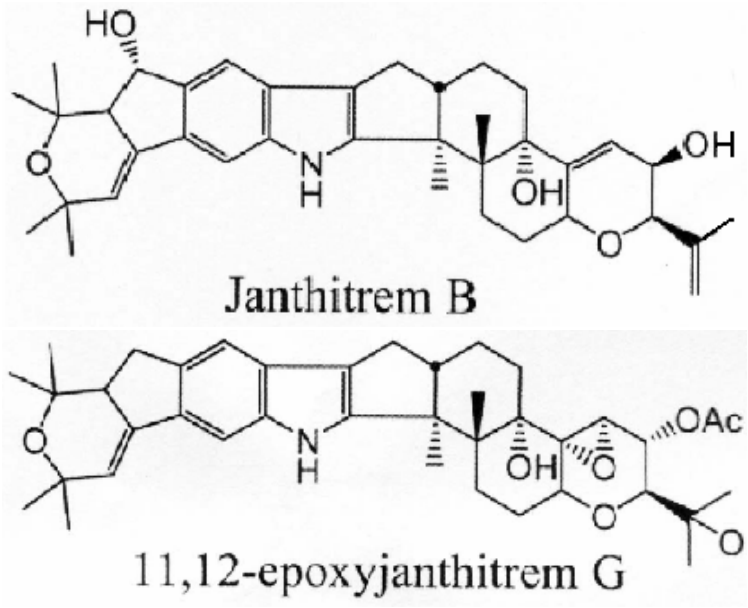
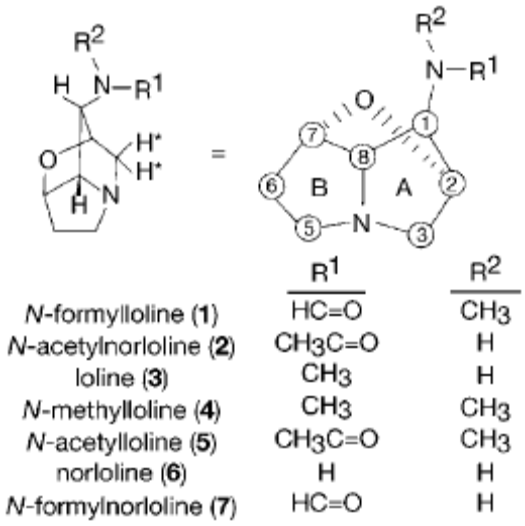


Figure 1.5. Structures of Janthitrem B and 11, 12-epoxyjanthitrem G (Tapper and Lane, 2004)

1.3.5. The loline alkaloids

Loline alkaloids are produced by several *Neotyphodium* and *Epichloë* species. They are almost exclusively found in grass-endophyte symbioses; outside of the grasses they have been identified in only a few plant species in the families *Fabaceae* and *Convolvulaceae* (Tofern et al., 1999). The loline group of pyrrolizidines, produced by endophytes, comprises saturated 1-aminopyrrolizidines with various sustituents on the 1-amino group

and an oxygen bridge between C2 and C7 (Blankenship et al., 2005) (Fig. 1.6). In contrast, most plant pyrrolizidines (necrines) have an additional carbon atom at C1 and contain neither the ether bridge between C2 and C7 nor the 1-amino group characteristic of the lolines (Blankenship et al., 2005). N-formylloline is usually the most abundantly produced *in planta* (Bush et al., 1997) and *in vitro* (Blankenship et al., 2001).



	R^1	R^2
N-formylloline (**1**)	HC=O	CH_3
N-acetylnorloline (**2**)	CH_3C=O	H
loline (**3**)	CH_3	H
N-methylloline (**4**)	CH_3	CH_3
N-acetylloline (**5**)	CH_3C=O	CH_3
norloline (**6**)	H	H
N-formylnorloline (**7**)	HC=O	H

Figure 1.6. The seven different loline alkaloids produced by *Neotyphodium* endophytes. Carbon number and ring assignments are indicated. (Blankenship et al., 2005)

Lolines can accumulate to extremely high levels in grass tissues. They have been shown to reach levels of more than two percent of the plant's dry weight in some associations between *N. uncinatum* and meadow fescue (Craven et al., 2001). These quantities far exceed the biomass of the fungus and the amounts of the other alkaloid groups. *N. uncinatum* tends to produce very high amounts of loline in planta compared to *N. coenophialum*. For example, U2 produces between 2000-9000 ppm when in associations with a range of different meadow fescue cultivars, whereas, MaxP only produces around 300 ppm in tall fescue cultivars (Cropmark Seeds Ltd., unpublished data).

Loline alkaloids produced by endophytes are potent, broad spectrum, insecticides (Riedell et al., 1991; Dougherty et al., 1998; Wilkinson et al., 2000; Meister et al., 2006). They exhibit a much broader range and more overt toxicity than does peramine (Riedell et al., 1991). The lethal dose rate (LD_{50}) for N-formyloline, N-acetylloline, and N-

methylloline is equal to that of nicotine sulphate for greenbug aphids (Schizaphis graminum) (Riedell et al., 1991). The effects of lolines are not limited to above ground insects as they are transported to, and can be readily detected in, the root tissue of the grass host (Cropmark Seeds Ltd., unpublished data) (Murphy et al., 1993; Koppenhofer et al., 2003; Lehtonen et al., 2005). They have also been shown to increase the resistance of the grass host to several fungal diseases (e.g. barley yellow dwarf virus) by way of deterring feeding from the insects that the fungal pathogens use as a vector to infect plants (Clarke et al., 2006; Lehtonen et al., 2006). Lolines have little or no antimammalian activity (Jackson et al., 1996) (Cropmark Seeds Ltd., unpublished data). Moreover, grasses infected with loline alkaloid producing endophytes, such as *N. coenophialum* and *N. uncinatum*, have greater tolerance to drought conditions (Arachevaleta et al., 1989; Bacon, 1993; Malinowski et al., 1997) than grasses infected with *N. lolii*, which do not produce lolines (Cheplick et al., 2000). Growth suppression (alleopathy) of neighbouring plants by meadow fescue infected with *N. uncinatum* (Bush et al., 1997; Malinowski et al., 1997) may indicate potential for additional beneficial roles of loline alkaloids in grass plant competitiveness and persistence (Spiering et al., 2002).

The biochemical pathway for lolines is, so far, unknown. On the basis of certain structural similarities of lolines with plant pyrrolizidines, it was proposed that lolines are produced by a pathway involving polyamines such as spermidine (Bush et al., 1993). However, results of precursor feeding studies have ruled this possibility out (Blankenship et al., 2005) and suggest that lolines are formed via a novel biosynthetic pathway from the amino acids L-proline and L-homoserine. Recently Spiering et al. (2005) identified two homologous gene clusters (*LOL-1* and *LOL-2*) associated with loline production in *N. uncinatum* (Spiering et al., 2005). Nine genes were identified in *LOL-1* and two in *LOL-2* along with another whose linkage to which cluster was undetermined. Most of these genes were regulated in *N. uncinatum* and *N. coenophialum*, and all were expressed concomitantly with loline biosynthesis (Spiering et al., 2005). The *lolC-2* gene in the *LOL-2* cluster was targeted in RNA-interference (RNAi) studies. A *lolC-2* RNAi construct was introduced into *N. uncinatum*, and in two independent transformants, RNAi significantly decreased *lolC* expression and loline alkaloid accumulation in culture

compared to vector-only controls, indicating involvement of *lolC* in the biosynthesis of lolines (Spiering et al., 2005).

1.4. Development of new/novel associations between *Neotyphodium* endophytes and grass hosts

Because of the agronomic significance of the relationship between endophytes and their hosts, a primary aim of endophyte research has been to develop new associations between important pasture species such as tall fescue, meadow fescue, and perennial ryegrass, and *Neotyphodium* endophytes, such that the benefits of infection (e.g., insect resistance) but not the disadvantages (i.e., livestock disorders) are conferred (Bouton and Easton, 2004). The creation of new associations between beneficial *Neotyphodium* strains and agronomically important grass cultivars is of considerable commercial importance to seed companies worldwide. This has resulted in specific strains of *Neotyphodium* endophytes that have no adverse effects on mammalian health now being commercially available in tall fescue in the United States (Max Q) and Australasia (Max P) (Bouton and Easton, 2004) and in perennial ryegrass in New Zealand (AR1) (Fletcher, 1999).

In New Zealand, 80% of all perennial ryegrass that is sown today contains AR1 endophyte. Therefore, the patent to AR1 endophyte is commercially very valuable to its owner, Grasslanz® New Zealand. Grasslanz® is a commercial subsidiary of AgReasearch New Zealand (a government owned and funded Crown Research Institute), and owns patents to strains of *N. lolii* (Endosafe, Endo 5, AR1, AR37, and AR6) and *N. coenophialum* (Max Q and P). Cropmark Seeds Ltd., which is in direct competition with Grasslanz®, holds the patent to a strain of *N. uncinatum* (U2). Cropmark Seeds Ltd. believes that U2 has significant agronomic potential if associations can be made between it and many of their patented and unpatented grass cultivars. Cropmark Seeds Ltd. has only recently begun to fund some research into *Neotyphodium* endophytes, whereas Grasslanz®, in conjunction with AgResearch New Zealand, has been doing so for several decades. This, along with the fact that *N. coenophialum* and *N. lolii* were discovered

before *N. uncinatum*, has resulted in there being much more published research on *N. lolii* and *N. coenophialum* than on *N. uncinatum*.

To create a new association between an endophyte and a grass host, the endophyte must be inserted artificially into the host. So far only two methods have been successfully developed to achieve a stable infection of the host. The 'micro-slit technique' involves making a 5 mm slit in the basal meristem of a grass seedling that has been grown in the dark for nine days, placing a small piece of endophyte in the slit, and placing the seedling back in the dark for another nine days before adapting it to the light (Latch and Christensen, 1985). The problems with this technique are that many of the hosts do not survive the procedure and that it has a very low success rate at achieving successful stable infections. Using this technique Latch and Christensen (1985) inoculated perennial ryegrass with *N. lolii* and tall fescue with *N. coenophialum* or *N. lolii*. Only 45-61% of the inoculated seedlings in these experiments survived the inoculation procedure and only 9-11% of the inoculated seedlings became successfully infected with endophyte (Latch and Christensen, 1985).

The second method, developed by Johnson *et al*. (1986), involved the inoculation of tall fescue with *N. coenophialum* by means of a callus culture (Johnson et al., 1986). Although this method achieved a higher endophyte infection success rate of 17%, it was very time consuming taking around 30 weeks to produce a small seedling (Johnson et al., 1986). For this reason it has not been used since it was developed to establish artificial plant-endophyte associations.

Very little is known about the host specificity of *Neotyphodium* endophytes. Studies have been conducted to gain insight into the host specificity of different species of *Neotyphodium* and *Epichloë* endophytes for different species of grass hosts. However, none of these studies have yielded any significant results (Koga et al., 1993; Leuchtmann and Clay, 1993; Christensen, 1995). It appears to be just as difficult to successfully inoculate a foreign host (i.e. tall fescue in the case of *N. lolii*) as it does to inoculate the natural host (i.e. perennial ryegrass) (Christensen, 1995). However, these same studies

showed that *N. coenophialum*, *N. lolii*, and *N. uncinatum* could all be successfully inserted, and form stable associations with tall fescue, meadow fescue, or perennial ryegrass (Christensen, 1995). Host genotype does appear to have some role in the establishment of artificial mutualistic associations as incompatibility has been observed on some occasions (Koga et al., 1993; Christensen, 1995). It is likely that host genotype has an affect on the alkaloid profile of the endophyte although the alkaloid profile is known to vary even in natural associations (Ball et al., 1995; Lane et al., 1997b; Easton et al., 2002). Host and endophyte genes involved in the association are presently the subject of much research (Tanaka et al., 2006; Zhang et al., 2006). Cropmark Seeds Ltd is working on the hypothesis that any problems involving host specificity of the U2 strain of endophyte will be able to be overcome by producing composite crosses (pers. comm., Nick Cameron, Cropmark Seeds Ltd.).

Floret inoculation has proven to be a successful method in the inoculation of *Triticum aestivum* (wheat) (Engle et al., 2003) and *Hordeum vulgare* (barley) (McCallum and Tekauz, 2002; Lewandowski et al., 2006) with *Fusarium graminearum*. Wheat and barley are also members of the grass family *Poaceae* and thus have similar floret morphology to perennial ryegrass, tall fescue, and meadow fescue. *F. graminearum* is a parasitic fungus that causes a disease known as *Fusarium* head blight or head scab in various cereal crops. *F. graminearum* spores infect a wheat floret, which is open for pollination, by germinating inside the floret and the mycelium growing down through the cells of the stigma into the ovary (Miller et al., 2004). This can result in either sterility of the ovary and no seed being produced or the seed filling poorly and thus poor quality seed being produced, ultimately resulting in yield loss (Engle et al., 2003).

Different methods of floret inoculation using liquid culture of *F. graminearum* have been used to examine the spread of the fungus throughout the floret (Miller et al., 2004) and to test the natural resistance of wheat cultivars to *Fusarium* head blight (Engle et al., 2003). Engle et al. (2003) used four different methods of floret inoculation to inoculate wheat florets with *F. graminearum*: a macroconidial suspension injection into the central floret on the spike using a hypodermic syringe; spraying a macroconidial suspension onto the

entire surface of the spike to the point of run-off with a hand atomizer; placing a drop of acrospore suspension on the junction of the glume, lemma, and palea of emasculated spikelets; and placing a drop of acrospore suspension on groups of anthers (Engle et al., 2003). The highest rate of infection (83.5% of all florets inoculated became infected) was achieved via the central floret injection (Engle et al., 2003).

Floret inoculation has also been used successfully in the genetic modification of *Arabidopsis thaliana* and other plant species with gene constructs transferred via the vector *Agrobacterium tumefaciens*. The three most commonly used floret inoculation techniques involve: (1) the developing floret being dipped in a suspension culture of *A. tumefaciens* (which has had the gene(s) of interest inserted into its T-DNA region) (Clough and Bent, 1998); (2) the suspension culture being directly injected into the developing floret with a pipette (Martinez-Trujillo et al., 2004); and (3) the floret being sprayed with the suspension culture (Chung et al., 2000). A marker gene (e.g. antibiotic resistance) is usually included in the gene construct so that transformants can be easily identified (e.g. by germinating the seeds on a selection medium containing the antibiotic). These methods do not require the large amounts of time, skilled labour, and costly laboratory equipment as do the other methods such as particle bombardment, plant tissue culture, and leaf disc transformation for genetically modifying plant species via *A. tumefaciens*.

The ovules and/or mega-gametophytes are the target for the transformation with *A. tumefaciens* (Desfeux et al., 2000). Originally it was thought that the *Agrobacterium* transformed the pollen or gained access to the ovary via the pollen tube. However, studies have since shown that this is unlikely as transformed ovules have frequently been observed (via the incorporation of the *gusA* reporter gene construct) in non-pollinated flowers (Ye et al., 1999) (Desfeux et al., 2000). In *Arabidopsis* flowers, the gynoecium typically develops as a ring of cells that protrude from the floral meristem (Bowman, 1994). This ring is surrounded by separate discrete mounds of cells that form stamens, and by young developing petals and sepals. As floral development progresses, the gynoecium extends to form a vase-like structure that remains open at the top. It is only at

a stage approximately three days prior to anthesis that the stigmatic cap forms over the top of the elongated gynoecium, sealing off the interior to form enclosed locules. This is in contrast to other plants such as soybean, where locule closure occurs more than ten days prior to anthesis (Johns and Palmer, 1982). *Arabidopsis* ovule primordia arise approximately one day prior to closure of the gynoecium, and megasporocyte formation is not estimated to occur until approximately one day closure of the gynoecium (Bowman 1994 book). In the large study conducted by Desfeux et al. (2000) transformants were only obtained from developmentally young flowers that were still five or more days from anthesis. Thus, it was hypothesised that no transformants were obtained from flowers that were more mature at the time of inoculation because these flowers carried closed locules, preventing access of *Agrobacterium* to the developing ovules and megaspores (Desfeux et al., 2000).

Floret inoculation has never been reported with regards to infecting a grass host with a *Neotyphodium* endophyte. A much less time and labour intensive inoculation technique would prove very useful in both the research and commercial areas of endophyte technology.

1.5. Endophyte growth, *in vivo* vs *in vitro*

Before it can be inoculated artificially into a host, endophyte has to be cultured on an agar medium. *In planta*, *Neotyphodium* endophytes grow almost as fast as the plant does whereas *in vitro* they have a very slow growth rate (Christensen et al., 2002). Reported *in vitro* radial growth rates for *N. lolii* colonies vary between 0.1-0.4 mm d^{-1} (Christensen et al., 1991), for *N. coenophialum* 0.3 mm d^{-1} (Morganjones and Gams, 1982), and for *N. uncinatum*, 0.25-0.35 mm d^{-1} (Gams et al., 1990; Blankenship et al., 2001) when grown on potato dextrose or malt extract agar containing 2% (w/v) agar. It appears that no one has ever attempted to investigate the reasons behind the difference between *in vivo* and *in vitro* growth rates, nor have ways of making *in vitro* cultures of endophyte grow faster been investigated.

The extremely slow growth rates of *Neotyphodium* cultures affects the rate at which all procedures that require *in vitro* culture of endophyte (e.g. inoculation and DNA extraction) can be performed. Most published studies on the morphology of *Neotyphodium* endophytes focus on their appearance *in planta* (White et al., 1993; Christensen et al., 1997; Christensen et al., 2002), whereas there are relatively few studies describing the appearance and morphology of these endophytes *in vitro*.

Another problem with *in vitro* cultures of *Neotyphodium* endophytes is that they seem to die for no apparent reason (Cropmark Seeds Ltd., unpublished data). This has been seen to happen with several cultures of endophyte in which the culture still looks alive to the naked eye but is, in fact, dead even though the colony has not covered the entire medium (Cropmark Seeds Ltd., unpublished data). The development of a simple viability test for *in vitro* cultures of *Neotyphodium* endophytes would help distinguish between viable and nonviable cultures and even parts of cultures.

Many different viability tests exist for other species of fungi. Succinate dehydrogenase (SDH) is a common respiratory enzyme involved in the tricarboxylic acid cycle. In arbuscular mycorrhizal fungi it reacts with nitro blue tetrazolium (NBT) resulting in insoluble formazan, which can be clearly distinguished (Vierheilig et al., 2005). Tetrazolium salt (2, 3, 5-triphenyltetrazolium chloride) dissolved in solution is also used as a seed germination indictor (Cottrell, 1947)). Initially the tetrazolium solution is water-soluble and colourless. The tetrazolium is reduced by respiratory enzymes (dehydrogenases), in the embryo of the seed, to triphenylformazon which is insoluble and red (Cottrell, 1947). Thus, if respiratory activity is present, which is presumed to be a sign of metabolism, the embryos will turn red and are considered viable. The darker the colour the greater the respiratory activity in the seed (i.e. light pink indicates a seed with reduced viability when compared to a seed that stains dark red) (Cottrell, 1947). As far as it is known neither of these staining techniques have been adapted for use as viability tests for *in vitro* cultures of *Neotyphodium* endophytes.

1.6. Stability of *Neotyphodium* endophytes in seed lots

A major, large scale problem at present with *Neotyphodium* endophytes is the stability of the endophyte infection in seed lots. In many areas of the world, novel, non-toxic endophytes are now considered to be essential components of sustainable grass-based pasture ecosystems (Hill et al., 2005). Consequently, the endophyte infection status (percentage and viability) is an important criterion of endophyte-containing grass seed quality. However, endophyte viability in seed can be short-lived and can change dramatically during the storage, transport, and distribution process, with viability reducing to 50-75% in seed lots not being uncommon (Barker et al., 2005). In some circumstances, a total loss of endophyte viability can happen. For instance, endophytes were detected in only 6.25% of the tall fescue plants derived from a seed lot that had earlier been determined to have a viable endophyte infection percentage of 80.7%, by immunoblot, histological and PCR methods (Dombrowski et al., 2006). For this reason, it is important that any endophyte test on seed be conducted within the final 2-3 months of storage (Barker et al., 2005). It is also critical to have a simple, rapid, and reliable method of detection to efficiently survey and monitor the endophyte infection status in grass seeds and plants. It would be particularly desirable if such a method could quantitatively determine the presence and the viability of endophytes simultaneously.

To date, several methods are available to detect *Neotyphodium* fungi in plants, including histological staining and microscopic examination of seed and plant tissue (Saha et al., 1988), serological methods such as ELISA tissue printing (Hahn et al., 2003) and immunoblot assay (Hill et al., 2002), and PCR protocols (Doss et al., 1998; Clement et al., 2001) . The microscopic and the immunoblot methods are well established and have been widely used for commercial seed testing. Microscopic examination has been shown to be a relatively reliable method of detection. However, it requires specific expertise and training to be able to identify fungal infections in tissue and seed. In addition, histological staining of fungal hyphae is a non-specific process that could result in false positives (Dombrowski et al., 2006). The immunoblot method can provide a quick, sensitive and high throughput means of detection when there is a need to examine large numbers of

individual seeds (Hill et al., 2002). However, this method may produce false positives caused by cross-reaction with proteins from the plant or closely related fungi (Hill et al., 1998). The immunoblot method displayed false positives as high as 20% in some Italian ryegrass seed lots (Dombrowski et al., 2006).

PCR is a rapid, sensitive and specific method allowing the detection of target DNA molecules in a complex mixture, and is widely used for plant pathogenic fungal diagnostics (Langrell, 2002). Numerous specific PCR primers with high discrimination from plant DNA have been developed for a wide range of fungal species (Martin and Rygiewicz, 2005). However, application of this technique for detecting fungal endophytes of the genera *Neotyphodium* and *Epichloë* in grass species has been limited to only a few studies. The most successfully used PCR method is the amplification of endophyte DNA from infected host tissue using primers specific to endophyte or fungi (Schardl and Siegel, 1993; Doss et al., 1998; Dombrowski et al., 2006). For this purpose, the ITS1/ ITS4 primers based on the ribosomal RNA internal transcribed spacer (Schardl and Siegel, 1993), the DNA fragments 11-1/11-2 (Doss and Welty, 1995), and IS-1/IS-3 primers based on the *β*-tubulin 2 gene (Doss et al., 1998; Clement et al., 2001), were developed and used to detect *Epichloë typhina* and *N. coenophialum* in tall fescue. Although Mirlohi et al. (2006) have successfully detected *Neotyphodium* spp in *Bromus tomentellus* using all these three sets of primers (Mirlohi et al., 2006), the applicability of these primers to other endophyte/host combinations remains to be tested. For instance, neither the IS-1/IS-3 nor the 11-1/11-2 primer pair was able to detect *Neotyphodium* endophytes from infected barley plants (Doss et al., 1998). More recently, Dombrowski et al. (2006) developed a new pair of endophyte-specific primers based on the *β*-tubulin gene (*tub2*) which were reported to specifically detect *Neotyphodium* endophytes in seeds and plant tissues of tall fescue, Italian ryegrass and perennial ryegrass (Dombrowski et al., 2006).

However, the data obtained with this conventional PCR methodology is strictly qualitative. Quantitative determination of the target fungus remains a problem (Alkan et al., 2004) (Isayenkov et al., 2004). Therefore, the application of conventional PCR

methodology to target DNA in large numbers of individuals, such as the determination of the viable endophyte infection percentage of a seed lot or plant populations, is both technically and economically impractical, because separate PCR reactions are required for each of the individuals to be tested. For this reason, the PCR assays cannot supplant established microscopic or immunoblot assays for endophyte detection in single seeds or seedlings, the methods currently used in seed testing laboratories (Dombrowski et al., 2006).

The development of the quantitative real-time PCR (qPCR) technique is a significant step forward in quantitative diagnostics, and the technique promises to be useful in plant pathology (Winston *et al*., 2002) and many other areas that require high sensitivity in both detection and quantification. This technique uses fluorogenic probes or dyes to detect the PCR product during the exponential phase of the cycle, which removes the limitations that characterise the endpoint detection methods used in conventional PCR (Alkan et al., 2004). Real-time PCR with gene-specific primer pairs and SYBR Green is able to quantify target DNA over six orders of magnitude, with a detection limit of one target DNA molecule in 1000 plant cells (Czechowski et al., 2004).

Very recently, the real-time PCR technique has been successfully used to quantitatively detect phytopathogenic and endophytic fungal species such as *Plectosphaerella cucumerina* (Atkins et al., 2003), *Botrytis cinerea* (Suarez et al., 2005), *Paecilomyces lilacinus* (Atkins et al., 2005), *Piloderma croceum* (Raidl et al., 2005) and *Tilletia caries* (Eibel et al., 2005) with very high sensitivity. The detection limit in these studies varied from 0.01-1 pg fungal DNA. For instance, Suarez et al. (2005) were able to reliably detect and quantify as little as 0.02 pg of *B. cinerea* DNA using the rDNA intergenic spacer (IGS) primers (Suarez et al., 2005). Such sensitivity would allow the detection of a single fungal spore in the sample (equivalent to 1 pg DNA) (Alkan et al., 2004).

The application of the real-time quantitative PCR technique has not yet been reported for the detection of *Neotyphodium/Epichloë* endophytes or any other fungal species in ryegrass or fescue. Furthermore, none of the current methods can distinguish between viable and nonviable endophytes in seeds, because the DNA, hyphal structure and proteins of the nonviable endophyte can remain intact in the non-germinated seed (Hill and Brown, 2000; Dombrowski et al., 2006).

To date, the only option to distinguish live from dead endophytes in seed is the "grow-out" test in combination with immunoblot detection or histochemical staining, which needs approximately six weeks to grow the seeds to be tested into 4-leaf-plants (Barker et al., 2005). A rapid and reliable *in planta* method to quantitatively determine the viable infection percentage of seeds and plant populations using real-time PCR has not yet been reported for any fungus and plant species.

1.7. Aims and Objectives

In the commercial grass seed/endophyte industry the slow growth rate of *Neotyphodium* endophytes *in vitro* is a problem because it limits the rate at which procedures such as the inoculation of seedlings with novel endophyte strains and DNA extraction can be performed. This in turn affects plant breeding programs and slows the development of new novel endophyte infected grass varieties which are the end product and earn revenue for the seed company. Surprisingly, very little research appears to have been done on the growth of *Neotyphodium* endophytes *in vitro* to date. Therefore, the aims of Chapter Two in this thesis were:

- To monitor the growth rate of *Neotyphodium* endophytes *in vitro* and assess the effect of two different medium types and concentrations on colony growth rate, appearance, and mycelial morphology with the aim of finding the medium that *Neotyphodium* endophytes would grow fastest on.

- To develop a simple, rapid, reliable method to test the viability of axenic *Neotyphodium* endophytes.

For a commercial seed company, producing a new novel endophyte-infected grass cultivar takes a lot of time and effort from plant/fungal biotechnologists, plant breeders, and their assistants, and therefore a substantial amount of capital investment. At present the most efficient way to create novel endophyte-desirable grass cultivar associations is via the micro-slit technique. This technique is slow, laborious, and has a low success rate of producing stable endophyte-host associations. If a method that was faster, less laborious and, most importantly, was more successful than the micro-slit technique could be developed it would be of significant benefit to the company that had knowledge of it. Therefore the aim of Chapter Three in this thesis was:

- To attempt to inoculate meadow fescue, and meadow fescue hybrids, with the U2 strain of *N. uncinatum* via floret inoculation.

Neotyphodium endophytes are unstable in the stored seed. Seed lots must be tested prior to sale to guarantee either a high endophyte infection rate or a nil infection. Thus, there is a market for the commercial testing of the viable endophyte infection rate of grass seed lots (pers. comm., Vaughan Ormsby, Cropmark Seeds Ltd). The methods currently used for commercial testing are very slow, laborious and costly. A faster, more efficient, low cost method for endophyte testing of seed lots needs to be developed. Therefore the aim of Chapter Four in this thesis was:

- To develop a rapid and reliable protocol for the determination of the viable *Neotyphodium* endophyte infection rate in seed lots of perennial ryegrass, tall fescue, and meadow fescue using real-time quantitative PCR. At the same time a method for the *in planta* detection of *Neotyphodium* endophytes using conventional PCR would be developed.

Chapter Two

Effect of the media on the growth rate and morphology of *Neotyphodium* endophyte colonies *in vitro*

2.1 Introduction

Growth of *Neotyphodium* endophytes *in vitro* differs significantly to that reported *in planta*. *In planta*, *Neotyphodium* endophytes are able to grow at almost the same rate as the grass host (Christensen et al., 2002). However, the fastest *in vitro* radial growth rates recorded for *N. coenophialum*, *N. lolii* and *N. uncinatum* colonies are around 0.3 mm d^{-1} (Morganjones and Gams, 1982; Gams et al., 1990; Christensen et al., 1993). The reasons for this large difference in growth rate between endophyte growing *in planta* and endophyte growing *in vitro* remain unknown.

The slow growth rate of *Neotyphodium* colonies *in vitro* can restrict the rate at which procedures that require *in vitro* culture (inoculation of seedlings, microscopic examination, and DNA extraction) can be performed. For this reason large numbers of cultures are usually kept. However, it can be difficult to maintain optimum conditions for so many cultures (checking for infection, sub-culturing etc.) and, when working with many different strains, a large amount of dark, temperature controlled space is required. *Neotyphodium* cultures have also been observed to die for no apparent reason (pers. comm., Nick Cameron, Cropmark Seeds Ltd). In large organisations it can be the job of one person to make sure that a large viable endophyte stock is maintained (pers. comm., Lester Fletcher, AgResearch NZ, Lincoln).

It is common practice to culture fungi on a 2% (w/v) agar medium (Smith and Onions, 1994). In most cases the slow growth rate of a fungus in culture is not a problem as most fungal species cover an entire culture dish within a week. Therefore, there has been no

need to investigate ways of obtaining faster growth of a fungal colony. Slow growing fungal species such as those of the *Neotyphodium* genus are rare compared to fast growing fungal species. Plant tissue culture is generally performed with media containing 0.7-0.8% (w/v) agar. This led to the hypothesis that the high concentration of agar in the medium was restricting the growth rate of the fungal mycelium. Assuming that it would take less energy for mycelia to abstract water and nutrients from, and to grow through, a less dense (lower percentage (w/v) agar) medium than a more dense medium; the radial growth rate of a *Neotyphodium* colony should be faster on a less dense medium.

The first aim of this part of the thesis was to monitor the growth rate of *Neotyphodium* endophytes *in vitro* and to assess the effect of different types and concentrations of media on colony growth rate, appearance, and mycelial morphology. A second aim was to develop a simple, rapid, reliable method to test the viability of axenic *Neotyphodium* endophytes.

2.2. Materials and methods

2.2.1. Isolation of endophyte strains

Neotyphodium strains were isolated from leaf sheath sections of their respective hosts (Table 2.1) by a slightly modified version of the isolation method described by Christensen et al. (1991). In a laminar flow cabinet, sections of leaf sheath, 5 mm long, were surface sterilised in absolute ethanol for 10 s followed by a rinse in bleach (Dynawhite® containing 0.4% NaOCl) for 2-5 minutes. 5 ml of penicillin-streptomycin solution (Sigma®) was added to 500ml MEA (Difco™ which, when made up to the manufacturer's recommendations, contains 1.5% (w/v) agar) when it had cooled sufficiently to be handled. The cooling medium was then poured into tissue culture dishes at a rate of 50 ml per dish. When the medium was beginning to set, the leaf sheath sections were submerged in it (3-4 sections per dish). If required the medium was sealed with a hot sterilised scalpel over the top of the leaf sheath section. Once the medium was

set, the plates were sealed with parafilm® and placed in a dark, 20^{o}C incubator. Mycelia generally emerged after 3-10 days from the ends of the leaf sheath sections but some took up to three weeks to emerge. After the mycelium had formed small colonies (1 cm in diameter), the colonies were sub-cultured to three or four MEA dishes, this time without the penicillin-streptomycin solution, and placed back in the incubator.

Table 2.1. *Neotyphodium* endophyte strains used in this thesis and their respective grass hosts from which they were isolated. *AR1 was not part of the growth rate experiment.

Endophyte strain	Endophyte species	Grass host species	Grass host cultivar
U2	*N. uncinatum*	*F. pratensis*	FP753
MaxP	*N. coenophialum*	*F. arundinacea*	Grasslanz Advance™
AR1*	*N. lolii*	*L. perenne*	Revolution™
WT (wild type)	*N. lolii*	*L. perenne*	Matrix™

2.2.2. The preparation of the media

Two types of media commonly used for culturing *Neotyphodium* endophytes were used for this experiment, potato dextrose agar (PDA) and malt extract agar (MEA), both of which were obtained from Difco™ laboratories. When made up to the manufacturer's recommendations these media contained the compounds shown in Table 2.2. The MEA and PDA were made up to three different concentrations based on the percentage (w/v) agar in the medium (Table 2.3). Note that a 1% (w/v) agar MEA or PDA medium refers to the fact that the final agar concentration is 1%, the other components of the medium varied depending on the specified agar concentration. For each treatment the MEA/PDA was dissolved in distilled water and then autoclaved at 121^{o}C for 15 min.

Table 2.2. The chemical compositions of the malt extract agar and potato dextrose agar when made up to the manufacturer's recommendations of a final agar concentration of 1.5% (w/v)

Malt Extract Agar (Difco™)		**Potato Dextrose Agar (Difco™)**	
Chemical	**Amount (g/L)**	**Chemical**	**Amount (g/L)**
Maltose	12.75	Potato starch	4.0
Dextrin	2.75	Dextrose	20.0
Glycerol	2.35	Agar	15.0
Peptone	0.78		
Agar	15.0		
Total	33.6	Total	39.0
pH	4.7 +/- 0.2	pH	5.6 +/- 0.2

Table 2.3. The amount of medium (g/L) compared to the concentration of agar (% (w/v)) in the medium for the six media treatments

Malt Extract Agar (Difco™)		**Potato Dextrose Agar (Difco™)**	
g/L medium	**% (w/v) agar**	**g/L medium**	**% (w/v) agar**
22.4	1.0	26	1.0
33.6	1.5	39	1.5
44.8	2.0	52	2.0

2.2.3. Setting up the experiment and recording the growth rate

There were ten replicates of each treatment for each strain of endophyte (U2, MaxP, and WT), making 180 plates in total. In a laminar flow cabinet, tissue culture dishes were filled with 40 ml of medium and left to set. A 5 mm^2 piece of endophtye was then taken from the outer region of an existing culture and placed in the centre of the dish. The plates were then sealed with parafilm® and placed in a dark 20^oC incubator. The maximum radius of each colony was measured and recorded every 5 d for a period of 40 d.

2.2.4. Biomass measurements, photography, and light microscopy.

At the end of the 40 day period after the last growth rate measurement had been recorded three of the ten plates in each treatment were placed in a 50^{o}C oven to obtain a dry weight (DW). Three control plates of each treatment (plates containing medium but no endophyte) were also placed in the oven as it was expected that the fungal mycelium would dry stuck to the medium, so the control measurement would need to be subtracted from each plate to obtain the DW of the mycelium. Three control plates were used to get an average DW of the medium. Three more plates/colonies were selected from each treatment based on the average appearance of all ten colonies in that treatment and photographed.

Finally, samples of mycelium were taken from the outer edge and centre of three of the cultures from 1% and 2% (w/v) agar MEA/PDA treatments. They were placed on a slide, stained with a drop of lactophenol cotton blue, and a cover-slip placed over top. Light microscopy was performed with an Olympus® BH-2 epifluorescence microscope with a Normansky differential interference contrast (DIC) attached. Images were taken using an Olympus® CoolSnap digital camera attached to the microscope.

2.2.5. Graphical analysis

Graphical analysis of the radial growth rate data was done using Microsoft Excel®.

2.2.6. Statistical analysis

The significance of the mean mycelial growth rate for each of the three strains was determined using the “least significant difference” (LSD) test.

2.2.7. Endophyte viability test

Three different solutions were used to test for the activity of succinate dehydrogenase.

I) Tetrazolium salt-succinate solution (10 ml)

2.5 ml tetrazolium salt (2, 3, 5-Triphenyltetrazolium chloride, BDH Laboratories) solution (4 mg ml^{-1})
2.5 ml Tris HCl buffer (0.2 M; pH 7.4)
1 ml sodium succinate (2.5 M)
1 ml $MgCl_2$ (5 mM)
3 ml distilled water

II) Tetrazolium salt solution (10 ml)

2.5 ml tetrazolium salt solution (4 mg ml^{-1})
7.5 ml distilled water

III) Nitro blue tetrazolium (NBT)-succinate solution (10 ml)

1 NBT tablet (Sigma®)
2.5 ml Tris HCl buffer (0.2 M; pH 7.4)
1 ml sodium succinate (2.5 M)
1 ml $MgCl_2$ (5 mM)
5.5 ml distilled water

A piece of endophyte was removed from a young (less than 30 d old) expanding colony of each of the four strains (U2, MaxP, AR1, or WT) and placed into test tubes, each containing one of the three solutions. The same was done with pieces of *in vitro* culture from the four strains of endophyte that were 4-5 months old and considered to be dead/non-viable. The test tubes were left overnight at room temperature and examined and photographed the next day.

2.3. Results

2.3.1. Radial growth rate vs type and concentration of media

The three different strains/species of endophyte U2 (*N. uncinatum*), WT (*N. lolii*), MaxP (*N. coenophialum*) all responded in different ways to the changes in the media. The growth rate of the U2 strain of *N. uncinatum* was most affected by changes in concentration of the medium. U2 colonies grew fastest on the 1% (w/v) agar media, followed by the 1.5% (w/v) agar media, and slowest on the 2% (w/v) agar media (Fig. 2.1 a, b; Table 2.4). The same trend was seen on both MEA and PDA (Fig. 2.1 a, b) though the difference between the radial growth rate of the colonies on the 1.5% and 2% (w/v) agar PDA media was not significant (Table 2.4). U2 colonies had a significantly faster radial growth rate on PDA than MEA at the 1% and 2% (w/v) agar concentrations (Table 2.4)

Table 2.4. Average growth rates of the three *Neotyphodium* strains on the different media. Within a species values followed by a different letter are significantly different ($P<0.05$).

Medium (all (w/v) agar)	**U2 (mm d^{-1})**	**WT (mm d^{-1})**	**MaxP (mm d^{-1})**
1% MEA	0.65^{b}	0.44^{a}	0.71^{d}
1.5% MEA	0.58^{c}	0.44^{a}	0.83^{a}
2% MEA	0.47^{d}	0.26^{c}	0.79^{bc}
1% PDA	0.70^{a}	0.43^{ab}	0.76^{c}
1.5% PDA	0.56^{c}	0.42^{ab}	0.81^{ab}
2% PDA	0.54^{c}	0.41^{b}	0.58^{e}

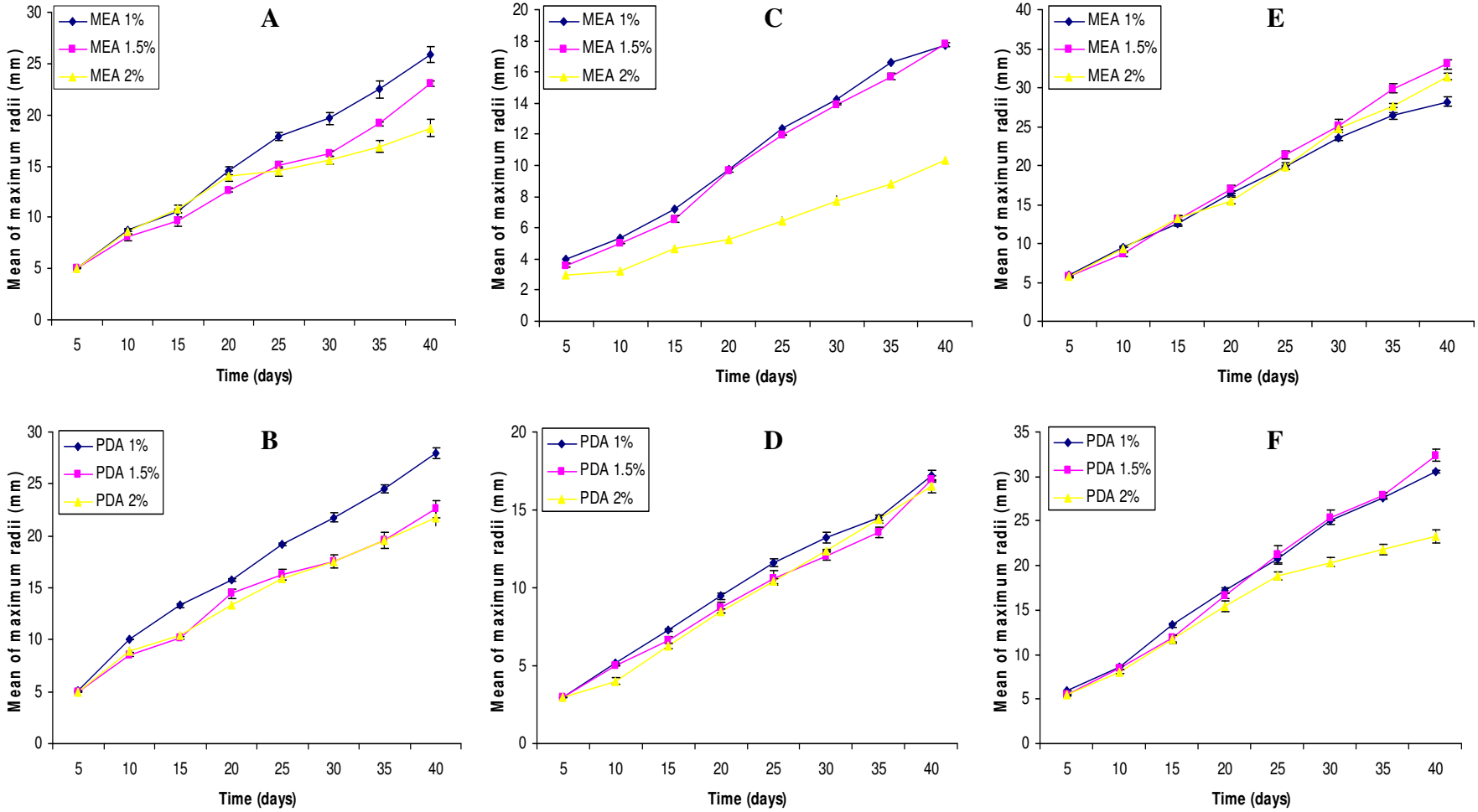


Figure 2.1. Growth of mean maximum length radii of U2 (A, B), WT (C, D), and MaxP (E, F) colonies on malt extract agar (A, C, E) and potato dextrose agar (B, D, F) media differing in concentration. Means (n=10) are indicated with error bars equal to ± 1 S.E.

The radial growth rate of the WT strain of *N. lolii* was only strongly affected by the 2% (w/v) agar MEA medium (Fig. 2.1 c, Table 2.4). The mean radial growth rate of the WT colonies on the 1% and 1.5% (w/v) agar MEA media was the same (0.44 mm d^{-1}) but was significantly slower on the 2% (w/v) agar media (0.26 mm d^{-1}). There was no significant difference between the radial growth rate of the WT colonies grown on PDA media (Fig. 2.1 d, Table 2.4).

Colonies of the MaxP strain of *N. coenophialum* grew fastest on the 1.5% (w/v) agar MEA and PDA media. On MEA, the radial growth rates of MaxP colonies on the 1.5% (w/v) agar media were followed by those on the 2% (w/v) agar media and finally by those on the 1% (w/v) agar media (Fig. 2.1 e). Although these differences were significant the range encompassing the three radial growth rates values was only 0.12 mm d^{-1}, whereas for U2 the range encompassing the same three values was 0.18 mm d^{-1} (Table 2.4) suggesting that the change in concentration of the MEA media had more affect on U2 than it did on MaxP. On PDA, the radial growth rate of the MaxP colonies grown on 2% (w/v) agar media was much slower than that of the colonies grown on the 1 and 1.5% (w/v) agar media (Fig. 2.1 f, Table 2.4). The radial growth rate of the MaxP colonies grown on the 2% (w/v) agar PDA media was similar to those of the colonies grown on the 1% and 1.5% (w/v) agar PDA media up until day 25 when it declined substantially (Fig. 2.1 f).

Overall, the MaxP strain grew the fastest followed by U2, with the WT strain having the slowest radial growth rate. A radial growth rate characteristic that was observed across all three endophyte strains that is not shown by these results was the 'boom and bust' cycle of growth. Individual colonies grew significantly one five day period but then little the next. This does not show in the graphs because they show the average growth of all ten endophyte colonies on the media.

2.3.2. Colony biomass vs type and concentration of media

Biomass measurements were unable to be obtained. The colonies did dry stuck to the media as expected. However, in most cases the dried media with endophyte stuck in them weighed less than the dried media without endophyte. This resulted in a negative biomass value.

2.3.3. Colony appearance vs type and concentration of medium

The colony appearances of the three different *Neotyphodium* strains/species were affected in different ways by the changes in type and concentration of the media. The U2 colonies grown on the MEA media had a waxy texture and were white in colour with an orange-pink tinge (Fig. 2.2 a-c). On the 1% (w/v) agar medium the colonies had a smooth flat outer region with a slightly raised central dome which had a small amount of aerial mycelium growing out of it (Fig 2.2 a). The colonies on the 2% (w/v) agar were almost completely domed, raised up to 7 mm above the medium surface, with an abundance of aerial mycelium (Fig 2.2 c). They did not have the large flat outer region that the colonies growing on the 1% (w/v) agar medium had. The colonies grown on the 1.5% (w/v) agar MEA media had an appearance in between these two descriptions, but somewhat slightly more like the appearance of the colonies on the 2% (w/v) agar MEA (Fig. 2.2 b).

All of the U2 colonies on the PDA media were brown in colour and were domed in shape, with the dome becoming more raised, but shrinking in diameter, as the concentration of the media was increased (Fig. 2.2 d-f). The number of white cottony aerial mycelium also increased as the concentration of the media was increased (Fig. 2.2 d-f).

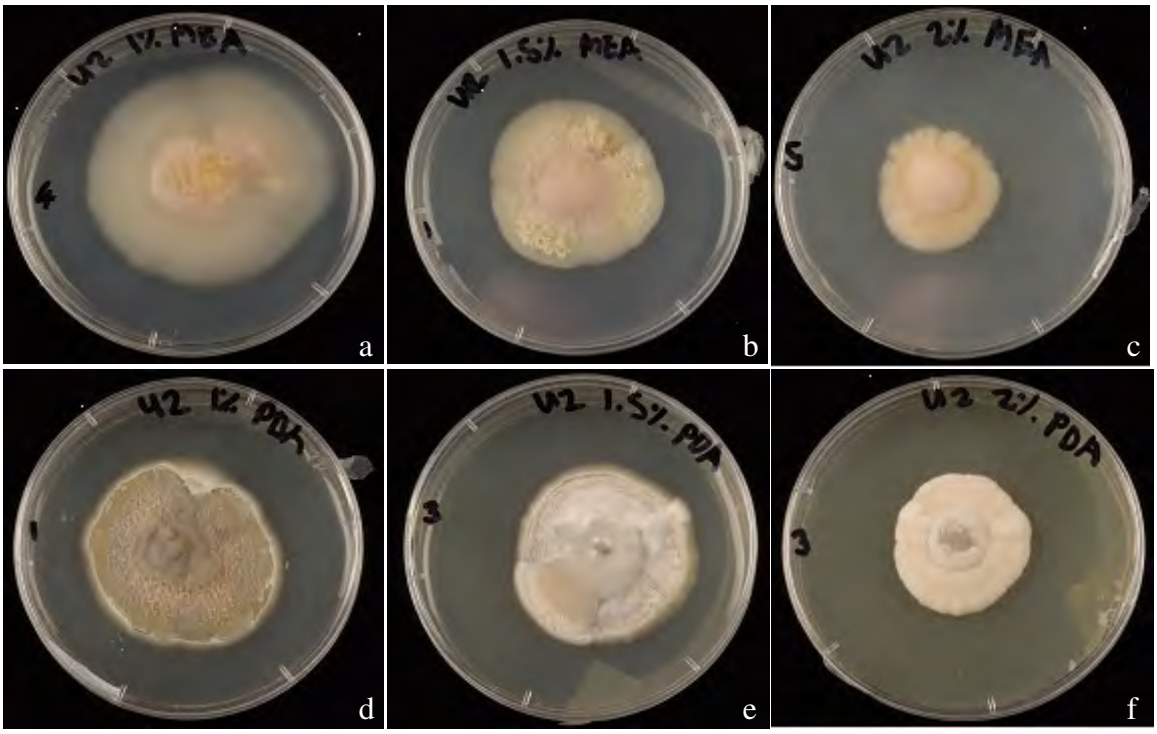


Figure 2.2. U2 colonies after 40 days growth on (a) 1% MEA, (b) 1.5% MEA, (c) 2% MEA, (d) 1% PDA, (e) 1.5% PDA, (e) 2% PDA

The change in media type and concentration affected colony appearance of MaxP (Fig. 2.3). On MEA, the MaxP colonies were white in colour and for the most part grew flat across the top of the media (Fig. 2.3 a-c). They did become slightly raised in the centre where what appeared to be 'folds' in the mycelial mass met with each other. These folds radiated out from the centre of the colonies and became more prevalent as the concentration of the media was increased (Fig. 2.3 a-c). The colonies grown on the 1% (w/v) agar media had a slimy-like appearance and no aerial mycelium (Fig. 2.3 a), whereas the colonies grown on the 2% (w/v) agar media were drier and did produce some aerial mycelium (Fig. 2.3 c). The colonies on the 1.5% (w/v) agar media had an appearance in between these two, but were more similar to the colonies on 1% rather than the 2% (w/v) agar media as they still had a slimy-like appearance and were without aerial mycelium (Fig. 2.3 b).

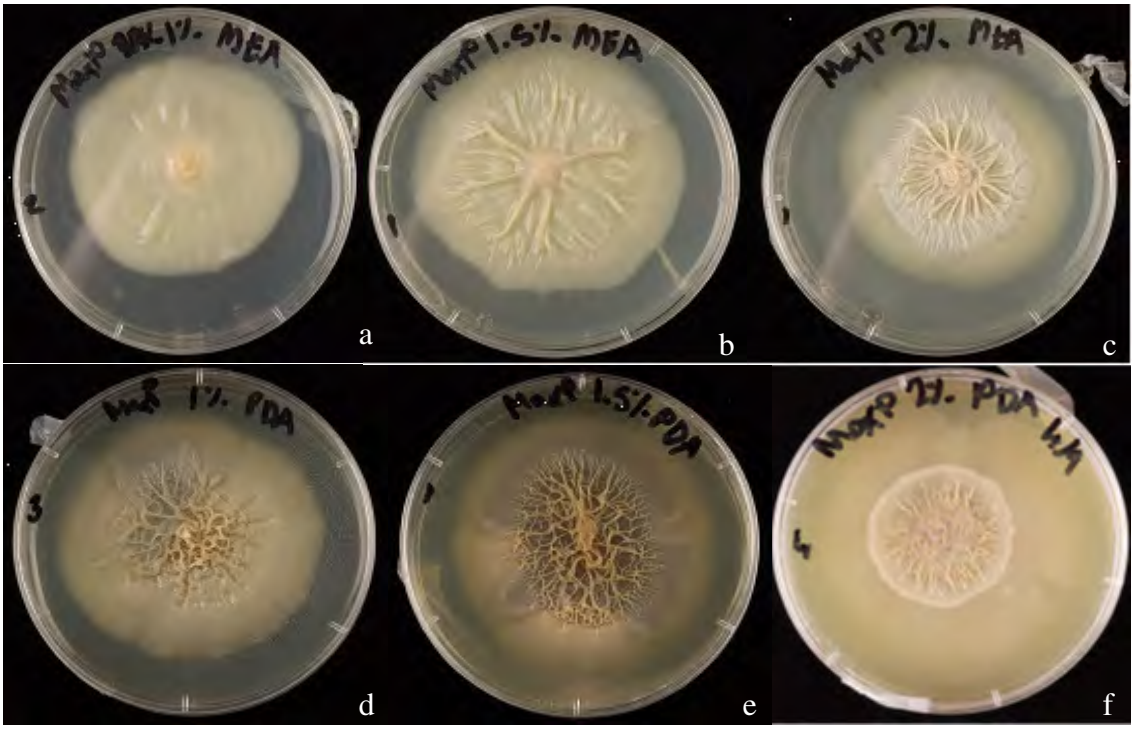


Figure 2.3. MaxP colonies after 40 days growth on (a) 1% MEA, (b) 1.5% MEA, (c) 2% MEA, (d) 1% PDA, (e) 1.5% PDA, (f) 2% PDA (photo (f) was taken by a different photographer; the colony was actually an even darker brown than the one in photo (e))

On PDA, sectoring of the MaxP colonies on the 1 and 1.5% (w/v) agar media was evident (Fig. 2.3 d, e). There were two different types of sectors. The first type of sector had a thick mycelial mat in the outer region and thick highly raised (6 mm) folds in the interior; this type of sector was dominant on the 1% (w/v) agar media (Fig. 2.3 d). The second type of sector had a much thinner outer region, the highly raised (6 mm) folds were still present in the interior although they were much thinner; this sector was dominant on the 1.5% (w/v) agar media (Fig. 2.3 e). The MaxP colonies on the 2% (w/v) agar mediums had a very unusual appearance (Fig. 2.3 f). They consisted of an inner colony that was predominantly flat except very fine folds extending out from the centre. This inner colony was surrounded by a large very thin flat outer region. Originally it was thought that the plates had become infected and that this outer region was a contaminant. Microscopic examination revealed that it was in fact endophyte mycelium. This outer region was not taken into account in the growth rate measurements as it was so thin that it

would not be able to be used efficiently for any of the procedures that require *in vitro* culture.

The colony appearance of the WT strain of *N.lolii* was also affected by the changes in type and concentration of the media (Fig. 2.4). On MEA, the colonies were white in colour with an orange-pink tinge. The colonies growing on 1 and 1.5% (w/v) agar grew flat across the top of the medium and had an abundance of fluffy aerial mycelia (Fig. 2.4 a, b). These colonies were circular in shape and had arc-like striations stretching out from the centre of them, forming a spiral-like pattern. The colonies grown on the 2% (w/v) agar MEA mediums were distinctly different from those grown on the 1 and 1.5% (w/v) agar media (Fig. 2.4 c). They were still predominately white in colour but had a more intense orange-pink tinge to them. They were a lot smaller and did not grow in the shape of a perfect flat circle like the colonies grown on the 1 and 1.5% (w/v) agar mediums. The colonies were raised, on average 5mm above the media surface, with steep sides and a brain tissue-like central area. These colonies had a predominately smooth waxy texture. Fluffy aerial mycelia were scarce although still present in some areas of the colonies.

On PDA, all of the WT colonies had a similar appearance. They were much the same colour as the WT colonies grown on MEA and once again the colour intensified as the concentration of the medium was increased (Fig. 2.4 d-f). They were circular in shape with a raised centre (3.5 mm) and had gently sloping sides stretching down to the media surface at the colony edge. All of the colonies demonstrated the spiral-like growth pattern described for the colonies grown on the 1 and 1.5% (w/v) agar MEA media. However, this pattern was more intense even on the 1% (w/v) agar PDA medium and became even more so as medium concentration was increased further (Fig. 2.4 d-f). All of the WT colonies on the PDA media were covered in fluffy white aerial mycelium.

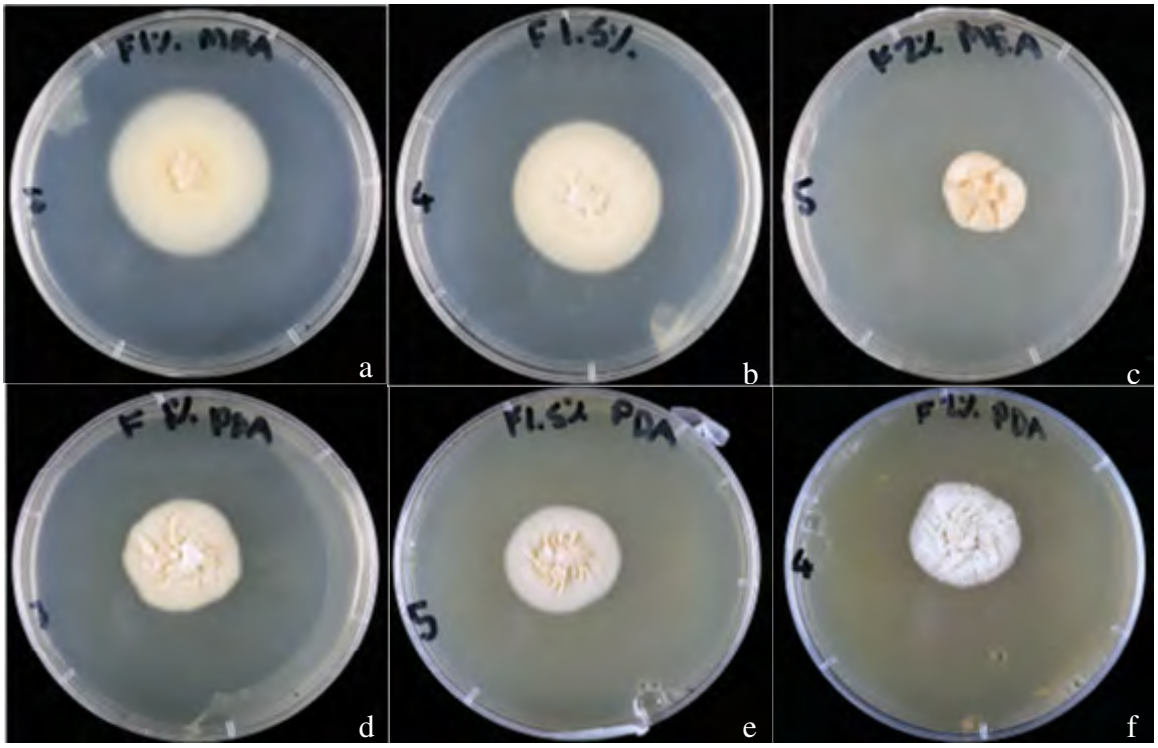


Figure 2.4. WT colonies after 40 days growth on (a) 1% MEA, (b) 1.5% MEA, (c) 2% MEA, (d) 1% PDA, (e) 1.5% PDA, (f) 2% PDA

2.3.4. Mycelial morphology

The effects of medium type on the mycelial morphology of the endophyte strains, as seen through a light microscope, were difficult to assess because characteristics of endophyte cultures, such as length of conidia, vary naturally. Mycelial morphology may also have been altered by the preparation of the slide (e.g. pressing down of the cover slip). However, observations were replicated (three cultures from each treatment examined) and all mycelial morphologies reported here were observed in all three of the cultures examined for that treatment. This makes it very unlikely that any of the reported mycelial morphologies were observed by chance.

There was a considerable difference in the observed mycelial morphology between the inner and outer regions of the U2 colonies grown on 1% (w/v) agar MEA medium. The

inner and outer regions of the U2 colonies grown on the other media did not differ as much in appearance (Fig. 2.2). Some of the hyphae from inner regions of the 1% (w/v) agar MEA colonies were group together into highly organised 'mega-strands' consisting of hundreds to thousands of individual hyphae growing close together, parallel to each other (Fig. 2.5 a). These mega-strands were observed in all of the U2 colonies on all of the media treatments suggesting that this may be a natural characteristic of U2 colony growth. In the smooth flat outer regions of the 1% (w/v) agar MEA colonies, unusual coiled hyphal structures were observed (Fig. 2.5 b). Much smaller rope-like mega-strands consisting of 5-10 individual hyphae were also observed in the outer region (Fig. 2.5 c).

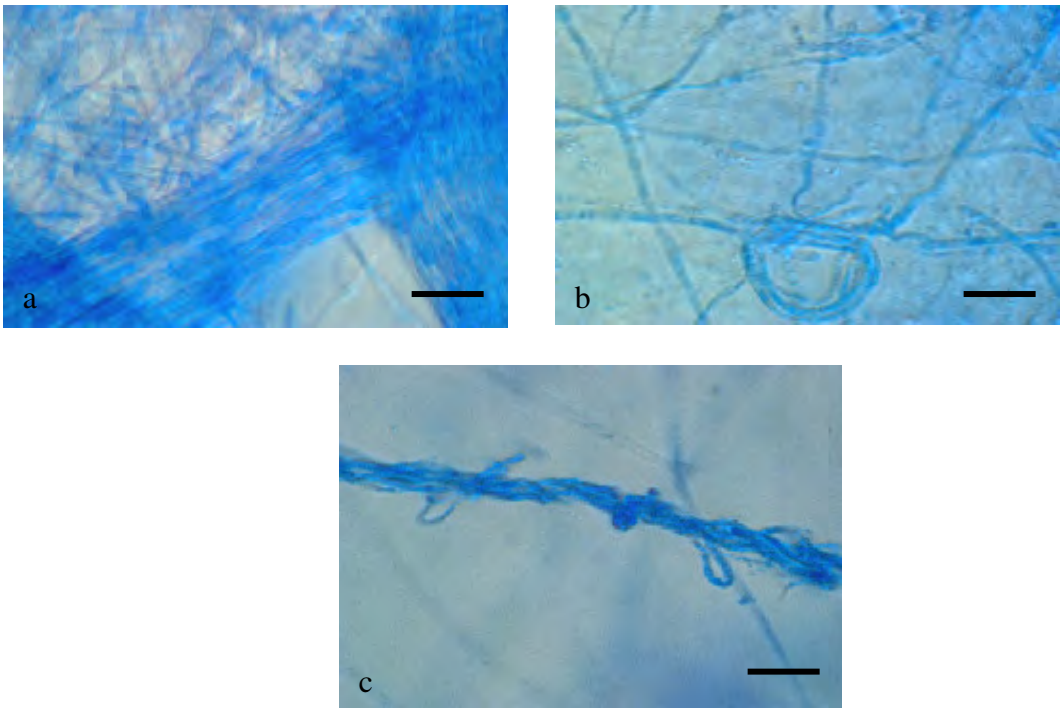


Figure 2.5. Mycelial morphology of U2 colonies grown on 1% (w/v) agar MEA. (a) The larger mega-strands observed in the inner region. (b) The coiled structures and (c) the rope-like mega-strands observed in the outer regions. Bars = 10 µm

The mycelial matrix in the inner regions of all the U2 colonies was denser, and more intensely branched than the outer regions independent of which media the colonies were

grown on. However, the type and concentration of the medium affected how big or small the inner/outer regions were. Branching of the hyphae was more frequently observed on PDA media (Fig. 2.6 a, b) than MEA (Fig. 2.6 c, d) media and its frequency increased as the medium concentration was increased.

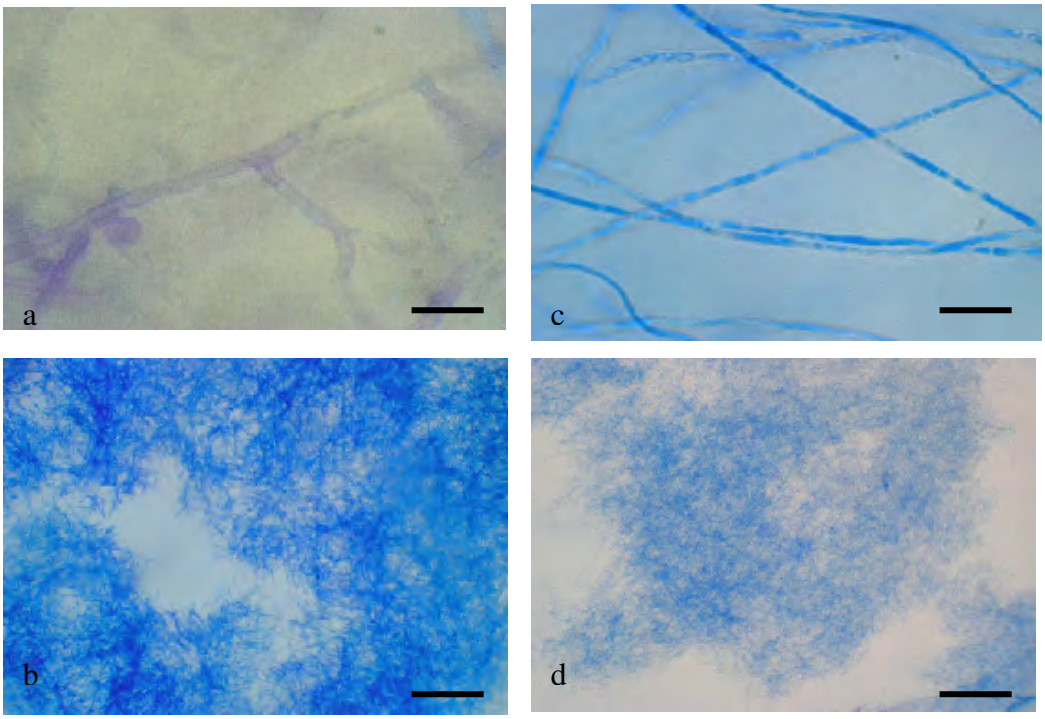


Figure 2.6. Outer regions of the U2 colonies grown on the 1% (w/v) agar (a, b) PDA and (c, d) MEA mediums. Bars = 10 µm (top) and 100 µm (bottom)

Conidia were rarely observed on either MEA or PDA media. They were most common in the inner regions of the MEA colonies, but were more or less uniform across the whole of the PDA colonies (these colonies didn't have distinguishable inner and outer regions). They were variable in length but generally between 10-25 µm. No spermatangia were observed.

The MaxP strain of *N. coenophialum* had a very different mycelial morphology from the U2 and WT strains. The colonies of MaxP on all the different media were characterised by intense sporulation. The MaxP colonies growing on the 1 and 2 % (w/v) agar MEA media had a similar mycelial morphology. The inner region was a very dense mycelial

mat. The hyphae were densely packed together with most aligned parallel to each other, and radiating out from the centre of the colony (Fig. 2.7 a, b). This morphology made it difficult to get good pictures of individual hyphae in this region. However, some were obtained and from these it could be seen that branching of hyphae and sporulation was still taking place in the inner regions of these MaxP colonies.

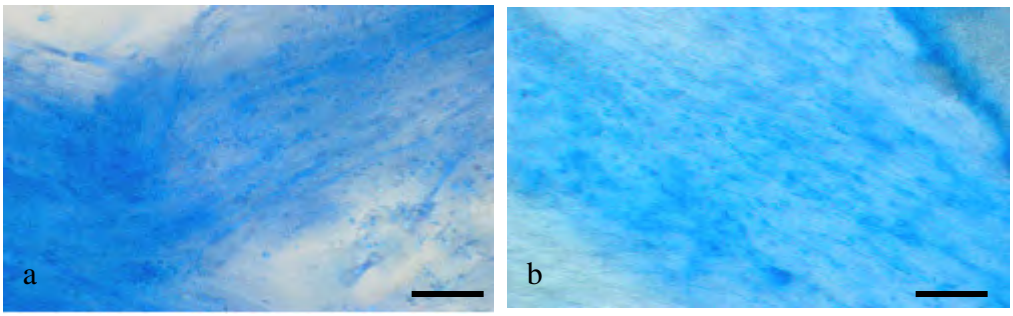


Figure 2.7. The dense mycelial morphology of the inner regions of the MaxP colonies grown on (a) 1% and (b) 2% (w/v) agar MEA. Bars = 10 µm

It was much easier to take quality images of the mycelial morphology in the outer regions of the 1 and 2% (w/v) agar MEA MaxP colonies. In this outer region of both the 1 and 2% (w/v) agar MEA media a much less dense mycelial matrix was present. A lot more branching was observed in this region along with many conidia. The conidia were circular or ellipsoid in shape with an average diameter of 20 µm and all were filled with spores, except for conidia which had burst open and released their spores (Fig. 2.8 a, b). The release of spores from the conidia was most frequently observed on the 1% (w/v) agar MEA media.

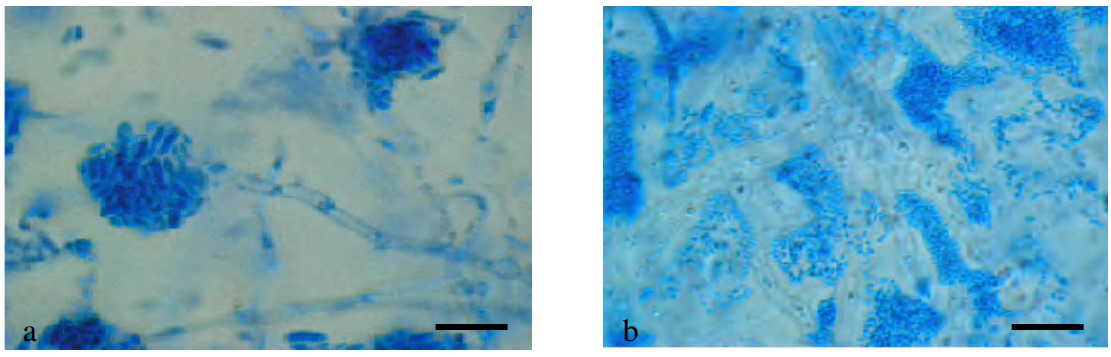


Figure 2.8. (a) A conidium observed in the outer region of the MaxP colony growing on the 2% (w/v) agar MEA medium. (b) Branching hyphae growing through masses of spores observed in the outer region of a 1% (w/v) agar MEA MaxP colony. Bars = 10 µm

On PDA, the mycelia in the inner regions of the colonies growing on both the 1 and 2% (w/v) agar media formed a matrix. Once again the high concentration of hyphae in the inner region coupled with dense sporulation made it very difficult to get quality images. On the 1% (w/v) agar PDA media, the matrix simply got denser from the outer to the inner regions of the colony, where mega-strands were once again observed. Conidia were observed in both regions. The very thin outer region of the colonies grown on the 2% (w/v) agar PDA medium was made up of sparsely spaced hyphae (Fig. 2.9 a). The inner region was a mycelial matrix; very dense sporulation was also observed (Fig. 2.9 b).

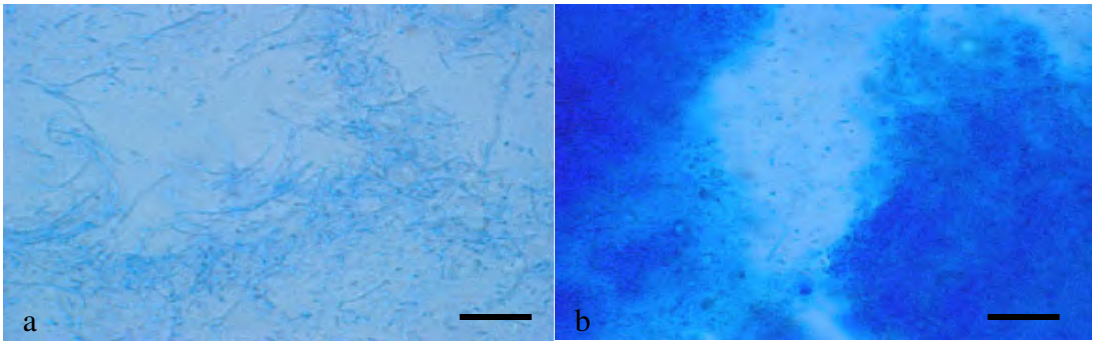


Figure 2.9. (a) Outer and (b) inner regions of MaxP colonies grown on the 2% (w/v) agar PDA media. Bars = (a) 10 µm and (b) 100 µm

The colonies of the WT strain of *N. lolii* had a similar mycelial morphology to U2 even though they were quite different in overall appearance. On PDA, the colonies grown on the 1 and 2% (w/v) agar media had a very similar mycelial morphology. The outer region was a loose mycelial matrix (Fig. 2.10 a) which allowed for some high quality images to be captured. Conidia measuring between 6-20 µm in length were observed along with the rope-like mega-strands seen in the U2 colonies (Fig. 2.10 b). Branching of hyphae was also readily observed (Fig. 2.10 c).

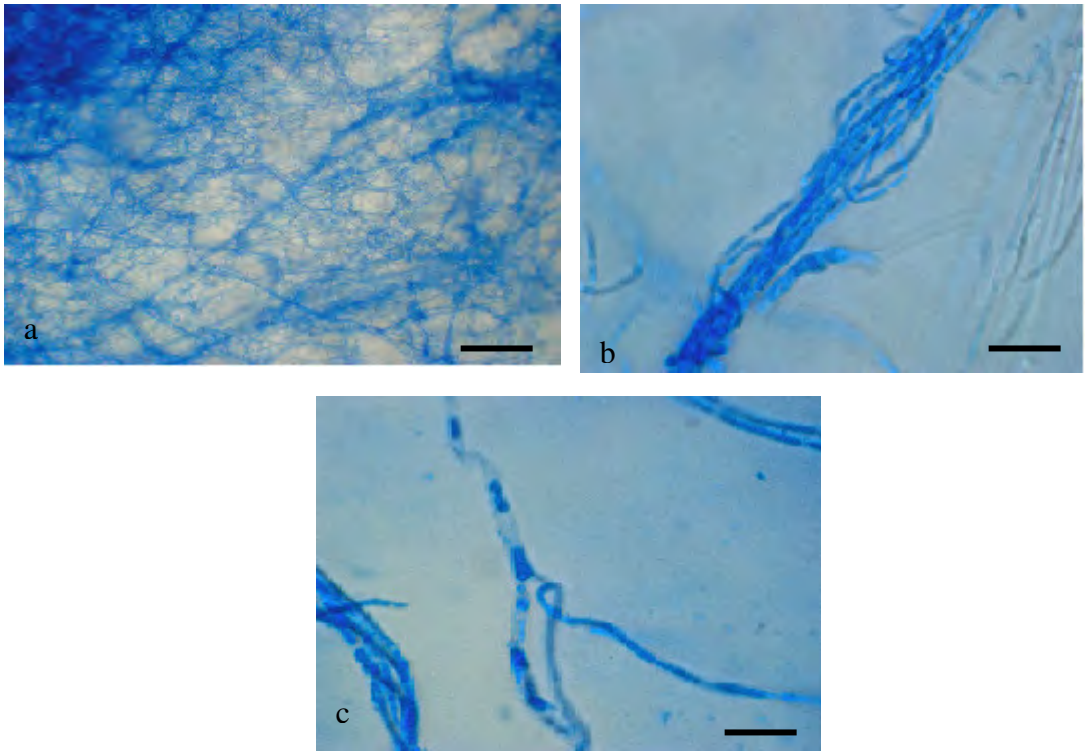


Figure 2.10. Mycelial morphology of the outer regions of the WT colonies grown on the 1 and 2% (w/v) agar PDA media. (a) Mycelial matrix of the 1% (w/v) agar colony. (b) Rope-like mega-strands. (c) Hyphal branching. Bars = (a) 100 µm and (b, c) 10 µm

On MEA, the mycelial morphology of the colonies grown on the 1% (w/v) agar medium was markedly different from those grown on the 2% (w/v) agar medium. This went along with the marked difference in overall colony appearance reported in Section 2.3.3. The outer regions of the colonies grown on the 1% (w/v) agar MEA media were once again a loose mycelial matrix but were even looser than that observed on 1% (w/v) agar PDA. Conidia and branching were observed much like that seen on the PDA media (Fig. 2.11 a, b). However, the rope-like mega-strands were absent. The mycelial matrix simply got denser in the inner regions of these colonies.

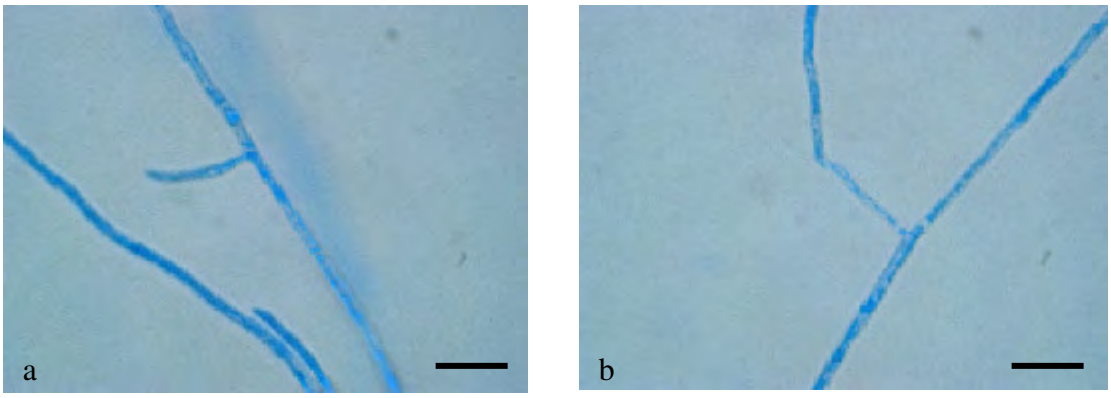


Figure 2.11. (a) Conidia and (b) hyphal branching observed in the outer regions of the WT colonies grown on the 1% (w/v) agar MEA mediums. Bars = 10 µm

There was a large difference in the mycelial morphology of the outer and inner regions of the WT colonies grown on the 2% (w/v) agar MEA media (Fig. 2.12). The outer region consisted of long wavy strands (Fig. 2.12 a). Conidia were observed (Fig. 2.12 b) frequently but branching of the hyphae was not as evident as it was in the colonies grown on the other media examined. The mycelial morphology of the inner regions was in stark contrast to this. It was a dense mycelial matrix, consisting of readily branching hyphae (Fig. 2.12 c). A far greater amount of branching was observed for this strain on this medium than for any of the other strains and media. Some of the hyphae were even observed to double back and apparently join with themselves again (Fig. 2.12 d).

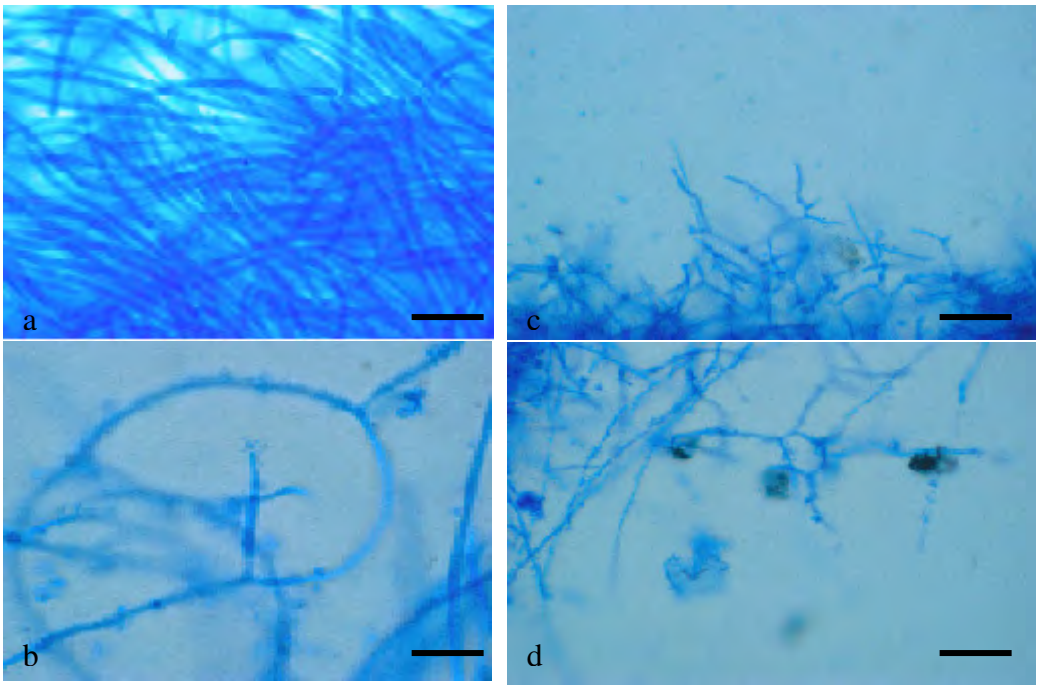


Figure 2.12. (a, b) The long wavy hyphae observed in the outer region and (c, d) the highly branched mycelial matrix observed in the inner region of the WT colonies grown on the 2% (w/v) agar MEA medium. Bars = 10 µm

2.3.5. Viability testing of *in vitro* endophyte cultures

All three of the tests for the viability of *in vitro Neotyphodium* colonies were successful in identifying viable mycelia from non-viable mycelia. The viable mycelia were either stained bright red, when tetrazolium salt was used as the active reagent (Fig. 2.13 a, c), or dark blue, when NBT was the active reagent (Fig. 2.13 e). In all tests the non-viable endophyte pieces remained unstained (Fig. 2.13 b, d, f).

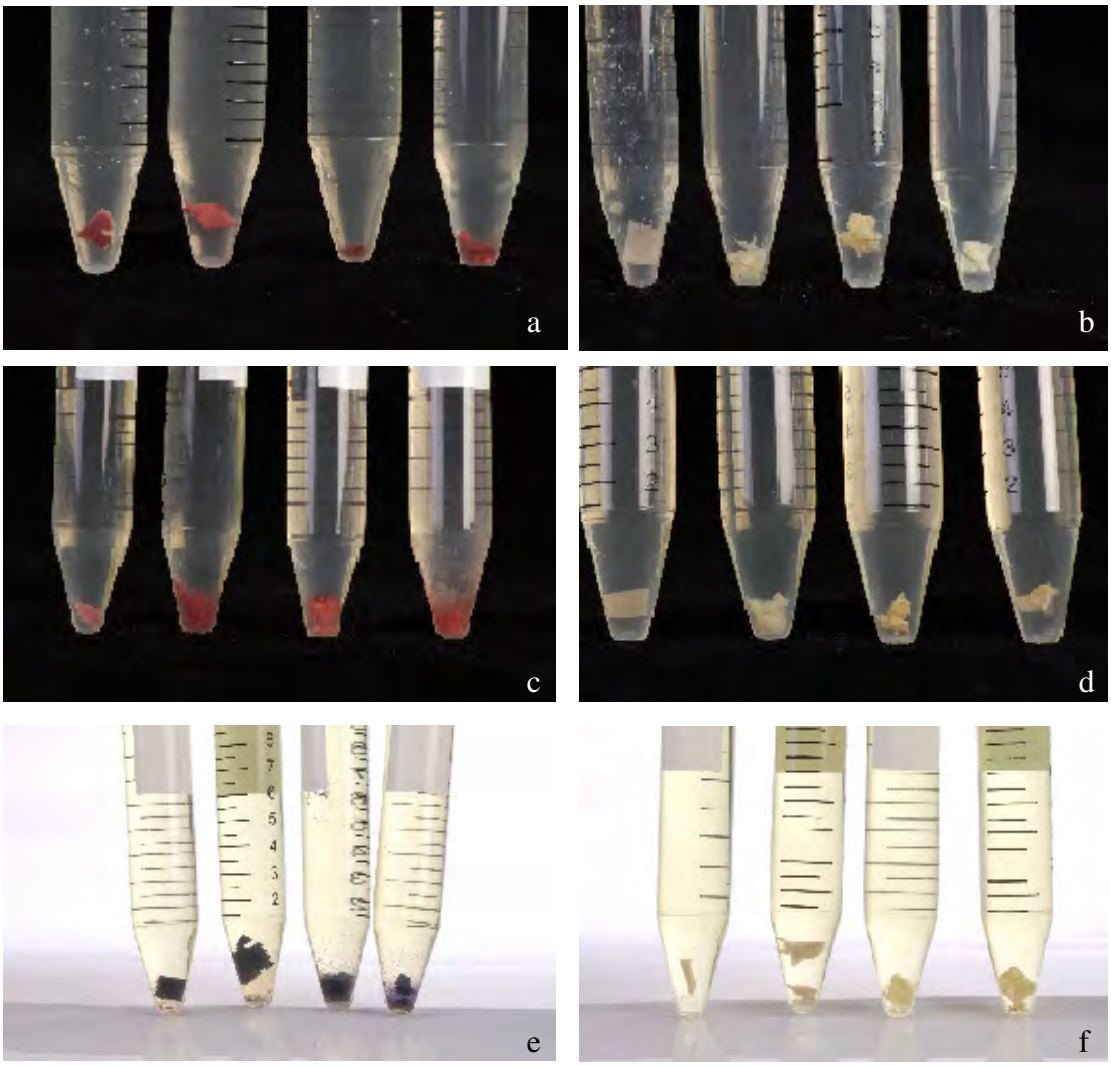


Figure 2.13. *In vitro* viability tests. (a, b) TZ-succinate solution, (c, d)TZ solution, (e, f) NBT solution. The viable (stained) endophyte is on the left, the non-viable (unstained) endophyte is on the right. Tubes contain pieces of U2, MaxP, AR1, WT endophyte from left to right in each image

In all three tests, the viable endophytes pieces taken from expanding colonies began to stain almost immediately after they were added to the test solution. However, during the experiment pieces of endophyte were also taken from mature colonies of the four strains that were six to eight weeks old and had stopped expanding and tested (data not shown). Mycelia from these colonies still became stained to the same intensity as mycelia taken from the expanding (less than four weeks old) colonies. However, it took two to four hours for these pieces of endophyte taken from mature colonies to begin to stain. There

was also variance in the time it took the mycelia to begin to stain between the different strains in the tests involving the mature colonies even in cases where the colonies were exactly the same age. Mycelia taken from the raised interior of mature U2 colonies growing on nitro-cellulose membranes also showed an interesting pattern of staining. The mycelium on the upper surface of the piece taken became stained while the mycelium on the underside (which would have been touching the nitro-cellulose membrane when in culture) remained unstained, suggesting that this mycelium on the underside is non-viable/dead (data not shown).

2.4. Discussion

2.4.1. The effect of medium type and concentration on the growth rate, colony appearance and mycelial morphology of *Neotyphodium* endophytes grown *in vitro*

To the best of my knowledge, this is the first time an experiment of this nature has been performed with *Neotyphodium* endophytes. The literature for other fungal species is also limited. The reason for this may be that the concentration of the medium is not really an issue when it comes to culturing most species of fungi as they are fast growing, so finding ways to increase their growth rate has never needed to have been investigated. However, in the case of slow growing *Neotyphodium* endophytes, a faster radial growth rate of *in vitro* cultures is advantageous for the use of this culture in procedures such as artificial inoculation, DNA extraction and sub-culturing.

On the whole there is very little published research on the growth of *Neotyphodium* endophytes *in vitro* especially when compared to what has been published about their growth *in planta*. The objective of the papers describing the growth of *Neotyphodium* endophytes was simply to provide the first taxonomic descriptions of different strains and species (Morganjones and Gams, 1982; Latch et al., 1984; Gams et al., 1990; Christensen et al., 1991). While a description of colony appearance and growth rate was provided, all microscopy in these papers tended to focus on the morphology of conidia as this at the time was thought to be a good characteristic by which to determine different strains and

species. Over time, as more and more endophyte strains were discovered, the morphology of conidia was shown to not necessarily be a good method for determining the species of a *Neotyphodium* endophyte (Christensen et al., 1991; Christensen et al., 1993). These early taxonomic descriptions (Morganjones and Gams, 1982; Latch et al., 1984; Gams et al., 1990) did not actually contain any photographic images of the hyphae or conidia, just simple drawings of the conidia, although more recently Christensen et al. (2001) presented some transmission electron microscope images of the conidia of *N. coenophialum* and *N. uncinatum*. The mycelial morphology of *in vitro* colonies seems to have been ignored or never published. In this thesis detailed microscopic observations of the mycelial morphology of *in vitro* colonies allowed the formulation of hypotheses into how different mycelial morphologies can affect the overall appearance and the radial growth rate of a colony, and how these were affected by changes in the medium.

Mycological experimental text books often contain recipes for many different media (Smith and Onions, 1994). Different media are known to induce the formation of aerial mycelium, conidia, and to increase the amount of sporulation (Smith and Onions, 1994). However, almost all of the recipes for media in mycological text books contain 2% (w/v) agar or greater (Smith and Onions, 1994). *Neotyphodium* endophytes have been cultured almost entirely on 2% (w/v) agar MEA and PDA (Morganjones and Gams, 1982; Latch and Christensen, 1985; Gams et al., 1990; Christensen et al., 1993; Christensen et al., 2000; Dombrowski et al., 2006). It seems as though researchers have simply followed the methods of the scientists that first isolated *Neotyphodium* endophytes. These scientists obtained their methods from mycological textbooks explaining the general culturing practices for all fungi. It was conveyed to me by a very experienced mycologist, when I first suggested my hypothesis, that changing the concentration of the medium would have no affect on the growth rate of the colonies (pers. comm., Ian Harvey, Plantwise, Lincoln) even though there seemed to be no published evidence to support this statement.

The results obtained from this experiment clearly demonstrate that the medium can have a major effect on the radial growth rate, colony appearance, and mycelial morphology of

Neotyphodium endophytes grown *in vitro*. The different strains/species in this experiment were affected differently by the changes in the medium parameters. This suggests that the overriding factor determining how *Neotyphodium* endophytes respond to changes in the growth media is simply that of genotype. Although each of the three strains examined in this experiment represent three different species of *Neotyphodium* endophytes, *N. uncinatum* (U2), *N. coenophialum* (MaxP), and *N. lolii* (WT), the results presented here should not be taken as indicative of how all of the strains from each of these species would respond to these changes in growth media. This is because past studies (Christensen et al., 1991; Christensen et al., 1993) and this one (Appendix 1) have shown that there is considerable variation in the morphological appearance of different strains of the same species. Past studies have concluded that determining the species of an endophyte based upon its colony appearance can often lead to false classification. Much of the early taxonomic confusion surrounding *Epichloë* and *Neotyphodium* endophytes was caused by this (Christensen et al., 1991; Christensen et al., 1993; Schardl and Siegel, 1993; Glenn et al., 1996).

The results from this experiment suggest that the mycelial morphology was affected by the changes in the medium and that this in turn affected the overall appearance of the colony but not always the radial growth rate. A good example of this is that every colony, within which highly organised hyphal mega-strands were observed, was raised. The results also suggest that if a certain medium type induces the formation of mega-strands, increasing the concentration of that medium will reduce the radial growth rate of the colony growing on it (e.g. U2 on MEA and PDA, MaxP on PDA, and WT on PDA). However, raised colonies do not always have mega-strands. It is also possible that there are strains with colony appearances different from those described in this thesis which possess mega-strands and would not respond in the same way as observed in this thesis. If the mycelia of a colony do not form mega-strands then how the colony appearance will respond to a change in the medium concentration cannot be predicted as it is dependant on the genotype of the strain. A change in the concentration of the growth medium could have little effect on the colony appearance and radial growth rate (e.g. MaxP on MEA) or it might have a dramatic effect (e.g. WT on MEA).

A thick, dry, and fluffy outer region of a colony is more desirable when it comes to inoculating seedlings, extracting DNA or sub-culturing the colony because it is simply easier to cut a piece of endophyte from the colony without any agar being attached to it. Agar compromises the techniques used for the inoculation of seedlings and DNA extraction. The slimy-like appearance of the MaxP colonies on the less dense MEA appeared to be related to the quantity of spores that had been released from conidia. During other parts of the project it proved very difficult to extract DNA from these slimy-like colonies, as agar was present in the slime, making these slimy-like colonies undesirable. Inducing the formation of reproductive structures is probable best avoided when culturing *Neotyphodium* endophytes for inoculation and DNA extraction procedures so long as it does not dramatically reduce the radial growth rate.

Although the results obtained from this thesis cannot be taken as representative for the species that each strain represents they may be somewhat representative of other strains of *Neotyphodium* endophytes, and even other fungal species, that have a similar colony appearance, independent of what species they are from. The original description for the colony appearance of *N. uncinatum* on 2% (w/v) agar MEA (Gams et al., 1990)fits well with what was observed in this experiment for the U2 strain on the same media even though it is extremely unlikely that these were the same strain. This would suggest that the growth rate of the *N. uncinatum* strain in Gams et al. (1990) could have been a lot faster than the 0.25 mm d^{-1} recorded (Gams et al., 1990) if this strain had been cultured on a 1% (w/v) MEA medium.

The first description of *N. lolii* (Latch et al., 1984) does not match that of the WT strain in this thesis. It was described as being tan in colour, lacking aerial mycelium, and having a radial growth rate of 0.13 mm d^{-1} on PDA at 25^{o}C (Latch et al., 1984). As this strain does not match the appearance of the WT strain it is unlikely that it would be affected by changes in the growth medium in the same way. However, the endophyte colony pictured in Figure 2 in Christensen et al. (1991) has a very similar appearance to the U2 colonies grown on the 2% (w/v) MEA. The colony pictured in Christensen et al. (1991) was grown on 2% (w/v) PDA and was originally isolated from perennial ryegrass. Restriction

fragment patterns at the time suggested that it was a strain of *N. lolii*, but the possibility of it being a strain of another *Neotyphodium* species was not completely ruled out (Christensen et al., 1991). Because of its very similar appearance to the U2 strain, this endophyte may also have demonstrated a faster radial growth rate if it too were cultured on a less dense medium.

The first description of *N. coenophialum* (one strain) in Morgan-Jones and Gams (1982) does not match that of the MaxP strain in this thesis; it is described as being covered in white cottony aerial mycelium (Morganjones and Gams, 1982). It is therefore unlikely that this strain would be affected by changes in the growth medium in the same way as MaxP was in this thesis. No other images or descriptions of *in vitro* colonies of *N. coenophialum* were able to be found in the literature for comparison.

The fact that the poorest radial growth rates for the MaxP and WT strains occurred on the 2% (w/v) agar PDA and MEA respectively is of interest because these two media are used in many papers, as well as being recommended by mycological textbooks, for culturing *Neotyphodium* endophytes and other fungal species (Smith and Onions, 1994). There are many published papers where the objective of the study was to artificially inoculate different strains of endophyte into different grass cultivars and then to observe characteristics such as hyphal morphology of the endophyte, alkaloid profile, and growth rate of the plant (Latch and Christensen, 1985; Koga et al., 1993; Christensen, 1995; Christensen et al., 1997; Malinowski et al., 1997; Christensen et al., 2000; Christensen et al., 2001, 2002; Zhang et al., 2006). These studies were mainly concerned with examining the host specificities of different endophyte strains and species as well as creating new novel associations between endophytes and grasses. These kinds of studies continue to be carried out today (Zhang et al., 2006; Scott et al., 2007). The results from this experiment suggest that many of these past studies may have been able to be completed faster if the optimal growth media were used for the isolation and culturing of the different *Neotyphodium* endophytes *in vitro*.

When the agar percentage of the medium was adjusted the concentrations of the other compounds (maltose, dextrose, potato starch, peptone, glycerol, and dextrin) in the medium were also altered. The alteration of the sugar concentration (maltose, dextrose) was the most important of these compounds as the other compounds were minor and are often not included in the descriptions of the components of either PDA and MEA found in mycological textbooks (Smith and Onions, 1994). The slowest radial growth rates for all three of the *Neotyphodium* strains were always observed on a 2% (w/v) agar medium. These media had the highest sugar concentrations. It would be expected that a higher sugar concentration would result in faster growth of the colony. The trends observed in this experiment were always the inverse of this suggesting that the agar percentage of the media was the major factor affecting colony growth. The possibility that the concentration of sugars in the media had an osmotic effect is also unlikely as the sugar concentration in the 2% (w/v) agar PDA and MEA was never more than 3% (w/v) which is not considered to be high (Smith and Onions, 1994).

Biomass of *Neotyphodium* colonies grown on a solid medium is not known to have been reported in any scientific study to date. This is probably because the radial growth rate of a colony is far more important than biomass when it comes to the sub-culturing of colonies. Observations during the viability testing of *in vitro* cultures also suggested that the upper surfaces of raised U2 colonies were more metabolically active, and therefore better for inoculation procedures, than the mycelial mass below. Results from this thesis suggest that some of this hyphal mass may in fact be dead. The best way to obtain biomass measurements of *Neotyphodium* colonies would be to grow them on nitrocellulose filters or use reverse agar which becomes liquid at 4^{o}C. However, nitrocellulose filters were used for culturing *Neotyphodium* endophytes in other parts of this project and they were observed to alter the appearance of colonies from what was seen on in this experiment. This change in colony appearance would probably have affected the biomass of the colony as well.

At the 6th International Symposium on Fungal Endophytes of Grasses (Christchurch, March 2007), intercalary extension was proposed as the primary mechanism responsible

for the large differences in growth rate between *Neotyphodium/Epichloë* endophytes growing *in planta* and *in vitro* (Christensen and Voisey, 2007). Fungal hyphae have always been thought to grow only at their tips. However, using a strain of *Epichloë festucae* transformed to express green fluorescent protein, Christensen et al. (2007) observed branching of hyphae in the expansion zone of ryegrass seedlings. This branching resulted in small groups of vertically aligned, still expanding plant cells being enclosed by a ring of hyphae. As the plant cells expanded, so did the hyphal ring (Fig 2.14). Thus, it was proposed that the pressure exerted on the hyphae by the expanding plant cells causes the hyphae to elongate and divide via intercalary extension. When hyphae were no longer amongst dividing or enlarging cells they ceased elongating and dividing but remained highly metabolically active (Christensen and Voisey, 2007). This mechanism of hyphal growth may be unique to *Neotyphodium/Epichloë* endophytic fungi (Christensen and Voisey, 2007).

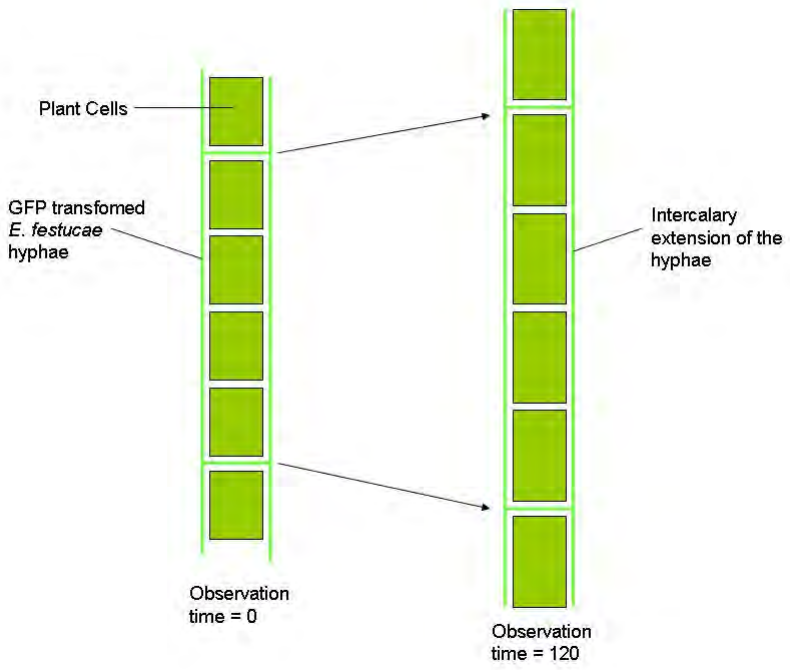


Figure 2.14. Intercalary extension of GFP transformed *E. festucae* hyphae observed in the expansion zone of a ryegrass seedling. Time is in minutes. Adapted from Christensen et al. (2007) presentation at the 6th International Symposium on Fungal Endophytes of Grasses.

2.4.2. Viability tests for *in vitro* cultures of *Neotyphodium* endophytes

The results showed that all three of the viability tests could clearly distinguish between viable and non-viable endophyte pieces. There appeared to be no difference in the time it took for the viable endophyte mycelia to stain bright red or the intensity of the stain between the tetrazolium salt-succinate solution (I) and the tetrazolium salt solution (II). This shows that the sodium succinate, Tris-HCl, and $MgCl_2$ included in the tetrazolium salt-succinate solution were unnecessary; the only reagent required to distinguish between viable and non-viable endophyte mycelium was the tetrazolium salt. The nitro blue tetrazolium tablets used as the active reagent in solution III were more expensive than the tetrazolium salt and did not dissolve rapidly in solution. The tetrazolium salt solution used in solutions I and II can be pre-prepared and kept for at least six months before it develops a red tinge itself. Therefore, the tetrazolium salt solution (II) was the most practical and efficient method for testing the viability of *in vitro Neotyphodium* endophyte colonies.

Time precluded a more rigorous investigation into the variance in the time it took the mycelia taken from mature colonies to stain. This was because cultures of the same age for each of the four species were not available. The observation that it took longer for the mycelia taken from the mature colonies that were in the so-called stationary phase (Prosser, 1995) to begin to stain indicates that the metabolic activity of the hyphae in these colonies had declined. *In planta*, hyphae in mature leaves also cease expanding but remain highly metabolically active (Herd et al., 1997; Tan et al., 2001).
The reduced metabolic rate in hyphae of mature colonies has been observed previously (Tan et al., 2001). Tan et al. 2001 used a strain of *N. lolii*, transformed with the *Escherichia coli* β-glucuronidase gene (GUS reporter gene system), under the control of a heterologous constitutive promoter (the *Aspergillus nidulans gpdA* promoter), to estimate the *in planta* distribution of endophyte metabolic activity (as GUS activity). *In vitro* colonies were also tested using this system. The expanding hyphae of growing colonies had high GUS activity whereas the GUS activity of hyphae in the mature colonies was a lot less (Tan et al., 2001). The results of the succinate dehydrogenase activity testing

described in this thesis, using tetrazolium chloride as the active reagent, compare well with what was seen by Tan et al. (2001). Expanding colonies had higher level of succinate dehydrogenase activity, suggesting a higher metabolic rate, than the colonies which had ceased expanding.

These results suggest that *in planta* hyphal extentension and metabolic activity can become uncoupled, with hyphal extension ceasing, while high metabolic rates are maintained (Tan et al., 2001). This should benefit the symbiosis, allowing the endophyte to utilise, in mature leaves, the equivalent biosynthetic capacity expended previously for rapid hyphal extension for the production of secondary metabolites (i.e. alkaloids) which protect the host (Tan et al., 2001). If undiminished biosynthetic capacity is indeed switched from biomass synthesis to secondary metabolite biosynthesis, the end of leaf extension should see the turning on of the respective pathways at high rates (Tan et al., 2001). The genes which control these secondary metabolite pathways are beginning to be revealed (Panaccione et al., 2001; Wang et al., 2004; Damrongkool et al., 2005; Spiering et al., 2005; Tanaka et al., 2005), which will allow the testing of this hypothesis.

However, the question still remains, why do colonies that have not yet apparently exhausted the agar stop expanding and eventually die? Colonies of all four strains (U2, MaxP, AR1, and WT) were at their maximum size after 6-8 weeks even though there was still plenty of un-colonised agar present in the plates they were grown on. It appears that the colonies stay in a stationary phase for around 10 weeks after they reach their maximum size, after which they begin to die.

What causes the colony to go into and remain in this stationary phase is unknown. Current suggestions include nutrient limitation, pH changes, and the accumulation of waste products (Prosser, 1995; Tan et al., 2001). Some of the metabolites synthesised by *Neotyphodium* endophytes may also be toxic to themselves, *in planta* such toxins might be dispersed away but when in a sealed plate may build up. In this experiment, when pieces of endophyte were taken from colonies in the stationary phase and subcultured to new media they began growing again and would stain immediately when tested with one

of the viability tests, indicating that the hyphal growth in the colonies that had reached the stationary phase was being limited by something.

The observation that U2 colonies may become non-viable from the base up suggests that it is something in media or amongst the mycelia that is limiting growth. Mature colonies in stationary phase also did not begin to grow again when the plate was opened then resealed. Gaseous products would be dispersed during this process, ruling them out as a limiting factor. The death of the hyphae that would seem to have access to the most nutrients first, and the rapid re-growth of the sub-cultured mature colonies, suggests that nutrient limitation may be the most probable cause for the stationary phase of growth. Nutrients and sugars in an agar medium are soluble and therefore follow nutrient gradients. In expanding *Neotyphodium* colonies the hyphae in the centre of the plate remain highly metabolically active up until the colony reaches the stationary phase. More nutrients may be required in the centre of the plate because highly metabolically active mycelia are present there for a longer amount of time during the expansion phase than areas further out from the centre. Therefore, nutrients and sugars in the media would be flowing down a nutrient gradient from the outer regions of the plate towards the centre. Because *Neotyphodium* endophytes are so slow growing, nutrients may be completely exhausted in the outer regions of the plate before the hyphae actually reach it, preventing growth to the edge of the plate. This does not happen with fast growing fungi because they reach the edge of the plate before the nutrients and sugars have been exhausted. A good way to test this hypothesis would be to sub-culture mycelia from an expanding colony to the un-colonised agar in the outer regions of a plate with a mature colony already present and see if it still maintains it rate of growth. However, the factors that cause *in vitro* colonies of *Neotyphodium* endophytes to go into a stationary phase of growth and then become non-viable remain unknown.

Chapter Three

New inoculation techniques for the artificial infection of grass hosts with *Neotyphodium* endophytes

3.1 Introduction

The low success rate of the inoculation techniques for establishing artificial associations between endophyte strains and a grass host is the major hurdle to the creation of novel associations for both research and commercial use. Currently, the predominant technique for the creation of artificial associations is the micro-slit technique (Latch and Christensen, 1985), which involves the insertion of a small piece of endophyte into the meristematic region of a 7-10 day old seedling. This technique is particularly stressful on the young seedlings being inoculated, as they are grown in the dark for 7-10 days on minimal media, inoculated via the micro-slit method, then put back in the dark for a further 7-10 days before being adapted to the light and planted out. This technique along with many unknown factors surrounding host specificity, results in a low success rate in achieving stable associations between the grass host and the endophyte. The average success rate of the micro-slit technique is around 20 – 30% for the infection of perennial ryegrass, tall fescue, or meadow fescue with either *N. coenophialum* or *N. lolii* (Latch et al., 1984; Christensen, 1995). The success rate of the technique with regards to the infection of these same grass species with *N. uncinatum* is much lower, being around 5% (Christensen, 1995).

Attempts at developing new techniques to improve inoculation efficiency have been made in the past (Johnson et al., 1986). Although these techniques were successful in achieving a stable infection of the host and had a similar success rate to the micro-slit technique, they took approximately ten times as long to produce a small infected seedling

(Johnson et al., 1986). For this reason, the micro-slit technique remains the most used in developing novel associations.

When using micro-slit technique as the method of creating artificial associations, the agronomic characteristics (e.g. root system, growth habits, and dry matter production) of the grass host cannot be selected for when the seedling is at such a young age. This presents a considerable problem to commercial plant breeders because it is impossible to control the agronomic characteristics of the plants that become successfully infected with endophyte. A plant with good agronomic characteristics and no endophyte infection can still easily out perform a plant with poor agronomic characteristics even if that plant is infected with endophyte (pers comm. Nick Cameron, Cropmark Seeds Ltd). Therefore, successfully inoculated plants with poor agronomic characteristics are not wanted by commercial plant breeders as it would take years of pair-wise crossing to improve their agronomic characteristics. An inoculation technique is needed in which the agronomic characteristics of the plants could first be examined, and then only the plants with the best agronomic characteristics inoculated.

Floret inoculation techniques have been used to achieve high success rates in both the infection of wheat spikelets with the pathogenic fungus *Fusarium graminearum* (Engle et al., 2003; Miller et al., 2004; Argyris et al., 2005) and the successful transformation of *Arabidopsis thaliana* seedlings with *Agrobacterium tumefaciens* (Clough and Bent, 1998; Chung et al., 2000; Martinez-Trujillo et al., 2004). These techniques involve spraying the floret(s) with suspension culture, dipping the floret(s) in suspension culture, or injecting suspension culture into the floret(s) with a pipette or syringe. Floret inoculation has not been attempted with *Neotyphodium* endophytes but it is known that they can be cultured in a liquid medium (Tan et al., 2001). In nature, the ovary of the developing seed is infected with endophyte at a very young age (Sugawara et al., 2004) but whether this means an ovary cannot become infected at a later date is unknown. The aim of this experiment was to successfully inoculate meadow fescue, and meadow fescue hybrids, with the U2 strain of *N. uncinatum* using different floret inoculation techniques.

3.2. Methods and Materials

3.2.1. Establishment of liquid culture

The U2 strain of *N. uncinatum* was isolated from its host and then sub-cultured on to malt extract agar (MEA) by the methods described in Section 2.2.1. Mature colonies (~6 weeks old) were lightly ground up with a mortar and pestle in a laminar flow cabinet. The ground up endophyte was then transferred to 1 L conical flasks containing 750 ml malt extract broth (MEB) (Difco™) or potato dextrose broth (PDB) (Difco™) and 50 mg L^{-1} chloramphenicol (Sigma™). The conical flasks were then placed on an orbital shaker in the dark at 26°C for two weeks. Subsequently, the liquid cultures were sub-cultured to 125 ml conical flasks and placed on a smaller orbital shaker under the same conditions. The cultures were generally ready for use in floret inoculation experiments after four weeks, although time did vary as to when they were actually used.

3.2.2. Plant lines

Three lines of meadow fescue (*Festuca pratensis*) and meadow fescue hybrids, provided by Cropmark Seeds Ltd., were used for this experiment (Table 3.1), to examine whether host genotype would have an affect on the success rate of the inoculation techniques.

Table 3.1. Seed lines used for floret inoculation experiments. Fp = *F. pratensis* (meadow fescue), Lp = *Lolium perenne* (perennial ryegrass), Lm = *Lolium multiflorum* (Italian ryegrass).

Seed line	Crossing history
FP673	Fp
FH722	(Fp x Lp) x Fp
FH780	(Fp x Lm) x Fp

The seed was first treated with Tilt® fungicide at a rate of 100 ul g^{-1} seed for five days to ensure the seed lines contained no endophyte. Seed from each line was then sown into germination trays and germinated in a glasshouse. The small seedlings were then placed

in a growth chamber, set to 8 h light at 20°C/16 h dark at 10°C, for one month to vernalise. After the vernalisation period, 36 individuals seedlings from each line were potted up into medium sized pots (15 cm in diameter) containing slow release potting mix (Appendix 2) and put in individual glasshouses. Twice, during the time the seedlings were growing aphids became came a problem; on each occasion the seedlings were sprayed with pesticide (Confidor®).

3. 2. 3. Floret inoculation methods

The florets of the seed heads were inoculated with liquid culture using two different methods. The first was a floret dip. Liquid culture was poured into a rectangular dish (25 cm x 8 cm) so that the solution was approximately 1 cm deep. The seed heads or spikes of the grass plants were then bent down so they were lying flat in the dish, and the liquid culture was washed back and forth over them. The second method was a central injection of liquid culture into each individual floret of the spike. This was done using a 200 µl pipette. The 200 µl pipette tip was cut back 4 mm to allow a more viscous solution to be administered. Approximately 50 µl of liquid culture was applied to each floret. After each spike had been inoculated it was marked with which method it had been inoculated by (by a post-it note being stuck to it) and tied up to a stick to prevent damage.

Both the floret dip and central injection techniques were repeated three times when the florets were at different stages. At each stage a set of six spikes were inoculated. The first set was inoculated when the spike had just reached maturity (i.e. it had stopped growing upward), the spikes were still green and 2/3^{rds} of the florets were closed or just beginning to open. The second set was inoculated five days later when 2/3^{rds} of the florets were open. The third set was inoculated another five days later when all florets were open and the spike was beginning to brown off. Each of the spikes were marked with which set they belonged too. Although each spike was not marked with exactly how old it was, when it came time to do another set of inoculations it was relatively easy to find spikes of a similar age since there were an abundance of them.

During inoculation two more methods were attempted in an effort to make the florets of the grass plants stay open longer as it was thought that this may increase the chances of a successful inoculation. The first method involved removing light from the developing spikes and isolating them from the surrounding environment, and thus pollen, by placing a brown paper envelope over six individual spikes (Fig. 3.1). When the florets of these spikes were fully open the envelope was removed and the spike inoculated via the floret dip method. The spikes were then left in the light to become fully mature. The second method was the inverse of this. Spikes were allowed to develop in the light to the stage at which the second set of inoculations took place. They were then inoculated in groups of three via the floret dip method and put in the dark (i.e. an envelope) to fully mature. This was done to three groups of three spikes.

Figure 3.1. The brown paper envelope used to restrict light to the spike (left) and the seed proof (but not pollen proof) plastic bags placed over the spikes post inoculation (right).

3.2.4. Seed collection, harvest, and re-sowing

Post inoculation, when the spikes were becoming mature, but prior to seed shed, a seed proof clear plastic bag was tied over the seed head to make sure no seed was lost (Fig. 3.1). When all spikes were fully mature they were cut off while still in the bag. Seed was harvested simply by threshing the seed heads between the hands and then picking up the seed with forceps and placing it in a container. The seed from each inoculation

treatment was then counted and re-sown into germination trays and placed in a glasshouse.

3.2.5. Histochemical staining for endophyte infection of grass seedlings

After four weeks growth, seedlings were examined via histochemical staining of a leaf sheath. A tiller was cut from each seedling and the outer leaf pealed off with forceps. The base of the leaf (i.e. the leaf sheath) was then cut off and placed flat on a microscope slide. The epidermis of the inner-side of the leaf sheath was then scraped with a scalpel. The leaf sheath was then covered in drops of lactophenol cotton blue, left for 1 min for the stain to soak in, and then a cover slip placed over the top. The leaf sheath was then examined under a microscope (Olympus CH-2) to check for the presence of endophyte.

Lactophenol cotton blue

0.8 g aniline blue
84 ml lactic acid
80 ml glycerol
50 g phenol
500 ml distilled H_2O

3.3. Results

The FH780 plants were the only ones to flower. Even though the FP673 and FH720 plants were vernalised for the same amount of time as the FH780 plants not one spike was observed. Thus, the floret inoculation experiment only involved the FH780 plants.

3.3.1. The growth of U2 in a liquid medium

U2 was observed to grow faster in PDB than MEB. This was interesting as U2 had previously been seen to do almost equally as well on malt extract agar (MEA) and it did on potato dextrose agar (PDA) (Section 2.3.1). The colonies in the PDB were also white

in colour (Fig. 3.2), not brown as had been observed on PDA (Section 2.3.3). The small pieces of U2 put into the liquid media grew into round ball-like colonies (Fig. 3.2).

Figure 3.2. The U2 strain of *N. uncinatum* grown in PDB (culture is 6 weeks old).

Large amounts of bacterial contamination were also observed when establishing liquid cultures of U2. Approximately 70% of all cultures became contaminated over the course of the four weeks. Some cultures even became contaminated before the endophyte was added. Little contamination was observed in the establishment of endophyte cultures on a solid medium, that were sub-cultured in the same laminar flow cabinet so it is unlikely the contamination got into the culture when the endophyte was added. The autoclave was known to malfunction several times during the preparation of the liquid cultures so this may have contributed to the high rates of contamination observed.

3.3.2. Comparison of floret inoculation techniques

Although seed was obtained from the inoculated plants and 53% (average across all techniques) of this germinated successfully, not one successful endophyte infection of a seedling was observed (Table 3.2).

Table 3.2. Success rates of the various inoculation techniques

Inoculation technique (set)	Number of spikes inoculated	Number of seeds harvested	Number of seeds that germinated/were examined	Number of successful infections observed
Floret dip (1)	6	164	86	0
Floret dip (2)	6	120	59	0
Floret dip (3)	6	133	65	0
Central injection (1)	6	141	77	0
Central injection (2)	6	145	81	0
Central injection (3)	6	125	70	0
Dark-Light Floret dip	6	85	43	0
Light-Dark Floret dip	9	169	95	0

3.4. Discussion

The results obtained from this experiment suggest that floret inoculation is not a feasible method for creating successful stable infections of grass hosts with novel strains of *Neotyphodium* endophytes. However, this was the first time an experiment of this nature has been conducted and many things were learnt from it. There were many factors, which were unknown at the beginning when planning the experiment, which may have had a large impact on the success of the experiment. Therefore, floret inoculation should not be completely ruled out as a feasible technique for establishing artificial associations between *Neotyphodium* endophytes and grass hosts.

The first problem with the methods used was the way in which the U2 strain of *N. uncinatum* grew in liquid culture. Although, it was known that *Neotyphodium* endophytes could be cultured in a liquid medium, it was unknown what the appearance of the growing endophyte would be in a liquid medium. *F. graminearum* sporulates and produces a liquid culture with a uniform appearance (Engle et al., 2003). In the case of U2, not many free, very small, pieces of endophyte were present after the four weeks of growth as had been anticipated. This colony appearance compromised the inoculation methods that were used for floret inoculation. Originally, another method of inoculation involving spraying the floret with the liquid culture as described by Engle et al. (2003) had been included in the experiment. This method was abandoned at this point, as it was simply not feasible, due to the U2 colony morphology in the liquid medium. The floret dip and central injection methods of floret inoculation were also compromised by the appearance of the U2 colony. Only a very limited amount of endophyte cells were sucked up by the pipette used in the central injection method and, thus, only a small amount of endophyte was inoculated into the florets. The same was true for the floret dip method, as there were not many free small pieces of endophyte in the inoculum the florets were dipped in. However, small pieces of endophyte were still seen on many florets after inoculation had been performed.

Another factor that could have affected the success rate of the inoculation techniques was the glass house conditions. The humidity was unable to be controlled in the glasshouse used for this experiment, and the large amount of growth chamber (humidity controlled) space that would have been required for this experiment was unavailable at the time. The low humidity in the glasshouses in the summer, when this experiment was done, resulted in the inoculum drying up on the spike very quickly. It is likely that the dry conditions would have killed the small pieces of endophyte before they got a chance to infect the floret. Growth and mist chambers, where humidity can be controlled, were used for the transformation of *Arabidopsis* seedlings with *A. tumefaciens* (Desfeux et al., 2000; Martinez-Trujillo et al., 2004) and for infection of wheat and barley spikes with *F. graminearum* (Engle et al., 2003; Jansen et al., 2005) to maintain a relative humidity of 60% or greater and prevent the inoculum drying out so quickly.

Addition of a surfactant or wetting agent to the inoculum may also have helped improve the inoculation success rate. Low concentrations (0.05% (v/v)) of Silwett L-77 in the case of *A.tumefaciens* (Clough and Bent, 1998; Desfeux et al., 2000; Martinez-Trujillo et al., 2004) or Tween 20 in the case of *F. graminearum* (Jansen et al., 2005) are commonly added to the inoculum to aid in its coverage of the florets. Although there were problems involving runoff of inoculum in some of the 1st set of inoculations, wetting the florets in the later inoculations (2nd and 3rd sets) did not seem to be a problem.

Being able to control the humidity and incorporating a wetting agent into the inoculum may have increased the success rate but this is unlikely. The difference in the liquid culture was probably the major factor responsible for no successful inoculations being achieved. Liquid culture of *F. graminearum* and *A. tumefaciens* are sporular and bacterial respectively, whereas the liquid culture of U2 was cellular. Trying to place small pieces of endophyte in the floret with forceps would be considered impractical, as it would prove to be a very difficult and strenuous procedure and most of the florets would likely be damaged and not even produce seed. Trying a similar experiment to this one using *N. coenophialum* would probably test the hypotheses that developing seeds can be successfully infected with endophytes a lot better as this species of endophyte has been observed to sporulate readily on agar media.

The physiology of the grass floret will also play a role in whether or not it is possible to artificial inoculate grass plants with endophyte via floret inoculation. In the case of *Fusarium* head blight the parasitic hyphae simply grow down between the cells of the stigma and into the ovary. It is unlikely a *Neotyphodium* endophyte would infect the ovary by this process as they are not parasitic and placing a piece of endophyte on tillers or leaves without wounding them does not result in infection of the host. However, this cannot be completely ruled out as developing stigmatic cells would be much softer than leaf of tiller cells and very recently *Neotyphodium* endophytes have been observed growing on the surface of inflorescences showing that in some circumstances they are able breach the epidermis of the host (Christensen and Saulsbury, 2007). If a *Neotyphodium* endophyte was to infect the developing ovary via the same mechanism as

A. tumefaciens infects *A. thaliana* the time of formation of the stigmatic cap would be the deadline for when a floret could be successfully inoculated (Desfeux et al., 2000). When the stigmatic cap forms in a developing grass floret is unknown but it is likely that it occurs before the stigma emerges from between the palea and the lemma (protective bracts enclosing the floret) making this mechanism of infection very unlikely. If it is possible to successfully infect developing seeds with endophyte via floret inoculation the most likely mechanism would be for the hyphae to grow down the pollen tube and into the developing seed.

The high rate of bacterial contamination observed in the liquid cultures was not completely unexpected as this problem has been reported before (pers. comm. Joshua Cobb, Brigham Young University). It is recommended that conical flasks be sealed with a cotton plug as well as autoclavable foil when establishing liquid cultures of endophytes and that the autoclaved broth is left for two days before the addition of the endophyte to make sure *in vitro* culture is not wasted. Different antibiotic solutions may also help prevent contamination of liquid cultures.

The large amounts of viable seed obtained in this experiment showed that these floret inoculation techniques caused little damage to the developing florets. Although there was no control treatment (no inoculation) the low germination rates were most likely the result of the growth conditions and the time of harvest and not the floret inoculation techniques. Floret inoculation of wheat with *F. graminearum* is used to evaluate resistance of individual plants and cultivars to *Fusarium* head blight. The techniques used in this experiment may be able to be used to evaluate the resistance of grass plants to various pathogenic fungi, such as *Claviceps purpurea* (ergot) and *Gloeotinia temulenta* (blind seed disease), which infect the developing florets and make the seed unviable. Both ergot infection of seed and blind seed disease have become more prominent in recent years and breeding grass cultivars resistant to them would be seen to be very desirable (pers. comm., Nick Cameron, Cropmark Seeds Ltd.)

Chapter Four

Determination of the viable endophyte infection rate in forage grasses: development of a rapid and reliable protocol using real-time quantitative PCR

4.1. Introduction

In New Zealand, it is critical that all developed pasture is infected with endophyte. The Argentine stem weevil, an exotic pest, has been observed to frequently destroy entire fields of pasture that are not infected with a protective endophyte (Rowan and Gaynor, 1986; Schardl et al., 2004). *Neotyphodium* endophyte infection does not spread rapidly throughout a pasture since this species does not possess a sexual cycle and reproduces solely by vertical transmission (White, 1988). Therefore, it is critical that seed lots in New Zealand are infected with a high percentage of viable endophyte.

In the U.S.A. and other parts of the world, endophyte infection is less critical with respect to the survival of the pasture. However, nil endophyte seed or seed infected with a novel endophyte is often still desired by farmers to prevent the detrimental effects that wild type endophytes can have on livestock and/or to give improved pest and/or drought resistance in the case of novel endophytes. Nil endophyte seed or seed which contains a novel endophyte is more expensive than normal seed (where the endophyte infection percentage or what species of endophyte the seed is infected with is often unknown) and, consequently, the consumer/farmer expects the seed to either contain nil or a high percentage of novel viable endophyte.

The problem that the seed breeding and seed marketing companies face is that *Neotyphodium* endophytes are unstable when in stored seed (Barker et al., 2005; Hill et

al., 2005; Dombrowski et al., 2006). There have been many cases where a seed lot that had a high viable endophyte infection percentage at harvest was found to have a much lower viable endophyte infection percentage months later (Barker et al., 2005; Dombrowski et al., 2006). Therefore, it is important that a seed lot be tested for its viable endophyte infection percentage 2-3 months prior to it being sown (Barker et al., 2005).

The current methods for determining the viable endophyte infection percentage of a seed lot are slow and labour intensive, which makes them costly for the seed companies. The aim of this experiment was to develop a rapid and reliable protocol for the determination of the viable *Neotyphodium* endophyte infection rate in seed lots of perennial ryegrass, meadow fescue and tall fescue using real-time quantitative PCR. At the same time, a method for the *in planta* detection of *Neotyphodium* endophytes using conventional PCR was developed.

4.2. Materials and Methods

4.2.1. PCR primer design

A BLAST search of GenBank® database was conducted for the *β*-tubulin (*tub2*) gene and ribosomal RNA internal transcribed spacer (*its1, its2, its3 and its4*) gene sequences with the aim of obtaining; 20-30 sequences (of either the *tub2* or *rRNA* gene) from the *Neotyphodium*/*Epichloë* endophytes (including *N. lolii*, *N. uncinatum*, *N. coenophialum*, *N. occultans*, *E. typhinia*, *E. festucae* and *E. bromicola*); 4-6 sequences from related non-endophytic fungi in the family *Claviceptaceae* (including *Claviceps purpurea* (ergot), *Cordyceps militaris* and *Acremonium stricta*); 8-10 sequences from fungal species known to cause grass seed/leaf diseases (including *Fusarium heterosporum*, *Microdochium nivale*, *Bipolaris sorokiniana*, *Magnaporthe grisea* and *Puccinia graminicola*); and 3-5 sequences from the host species (*Lolium perenne* (perennial ryegrass), *Festuca pratensis* (meadow fescue), *Festuca arundinacea* (tall fescue) and *Lolium multiflorum* (Italian/annual ryegrass)).

These sequences were then aligned using Clustal X (Thompson et al., 1997) to determine intron regions conserved among *Neotyphodium/Epichloë tub2* gene sequences but not between them and their close contaminant relatives. Forward and reverse primers were designed based on these conserved regions using Primer Premier 5.0. A BLAST search for each of the putative primer sequences was then conducted to ensure that they would not bind to any other fungal or plant DNA. These primer sequences, along with the IS-RS-5′ and IS-NS-3′ primer sequences designed by Dombrowski et al. (2006), were synthesised at Invitrogen®, New Zealand, diluted to 10 pmol/µl, divided into 100 µl aliquots and stored at -20^{o}C

4.2.2. DNA extraction

The four strains of *Neotyphodium* endophytes (U2, MaxP, AR1 and WT) isolated as described in Section 2.2.1 were sub-cultured onto MEA (Difco^{TM}) with a nitrocellose filter (Millipore®) on the surface prior to DNA extraction. The filter was used to prevent agar interfering with, and compromising, DNA extraction. Six contaminant species (three *Alternaria*, one *Epicoccum*, one *Stemphillium* and one *Fusarium*) which had been isolated from grass seed/seedlings by Nick Grbavac (Agriquality, Lincoln, New Zealand) and supplied in culture were similarly sub-cultured. *A. stricta*, another saprophyte commonly isolated from grass seedlings, is often misidentified as a *Neotyphodium* endophyte because of its similar appearance in culture. This happened during the course of this thesis and, as a consequence, it was also included in this experiment; it was cultured in the same way as the contaminant fungi. *C. purpurea* (ergot) is difficult to culture on agar, so DNA was extracted from ergot-infested nil endophyte seed supplied by Cropmark Seeds Ltd. DNA was also extracted from grass seed and seedlings known to be infected with the four different endophyte species (E^+ plants) (Table 1.1, Section 2.2.1) as well as from grass plants known to contain nil endophyte (E^- plants).

DNA was extracted from fungal and plant tissue using a modified version of the CTAB DNA extraction method. 100-200 mg of fresh tissue was ground under liquid nitrogen in a 1.5 ml tube with a plastic mortar. 600 µl CTAB extraction buffer was added to the tube,

which was vortexed to suspend the ground tissue in solution, and incubated in a dry block heater for 45-60 min at 60°C. The tube was centrifuged at 8000 rpm for 2 min and the supernatant was removed to a clean tube. 700 µl of chloroform was added, the tube shaken vigorously, and left to stand for 2 min. The tube was centrifuged again at 8000 rpm for 2 min and the upper aqueous phase removed to a clean tube. 700 µl of isopropanol was added, the tube mixed gently, and left to stand for 10-15 min to allow the DNA to precipitate. If there was a low yield of DNA the tube was centrifuged at 5000 rpm for 2 min. The upper part of the solution was removed with a pipette to leave the DNA filament and roughly 300 µl of solution. 500-1000 µl of 80% ethanol was added and the tube mixed gently to wash the DNA filament. The previous two steps were repeated 2-5 times until the DNA filament was white and clean. The tube was centrifuged at 4000 rpm for 2 min, the ethanol removed, and the DNA left to air dry for 5-10 min. The DNA pellet was re-suspended in 30-50 µl TE buffer or dH_2O depending on the yield. The concentration and purity of the DNA was checked using a Nanodrop® spectrophotometer so it could be adjusted to a concentration suitable (0.1-1 µg/µl) for efficient PCR. DNA was generally stored at 4°C as it was used soon after it had been extracted. However, excess DNA was often extracted so this was stored at -20°C.

Cetyltrimethylammonium bromide (CTAB) extraction buffer (40 ml)

0.8 g CTAB
0.4 ml Polyvinylpyrrolidine
11.2 ml NaCl (5 M)
4 ml Tris/HCl (1 M)
1.6 ml EDTA (500 mM)
22.4 ml distilled H_2O

4.2.3. Primer testing using conventional PCR

The eight different primer pairs (excluding the IS-RS-5′ and IS-NS-3′ primers) were first tested on pure endophyte DNA from each of the four endophyte strains (U2, MaxP, AR1 and WT) to ensure that they produced a single product. Conventional PCR was performed using a Faststart Taq DNA polymerase kit (Roche Diagnostics). The

composition of the PCR reaction volume is shown in Table 4.1. A negative control (DNA replaced with H_2O) was included in each run.

Table 4.1. Components of the PCR reaction volumes (20 µl in total)

Chemical	**Volume (µl)**
Distilled H_2O	9.8
10x PCR buffer (+Mg)	2
dNTPs (2 mM)	2
$MgCl_2$ (25 mM)	1
Forward Primer (10 pmol/µl)	2
Reverse Primer (10 pmol/µl)	2
DNA (0.1-1 µg/µl)	1
Taq polymerase (5 U/µl)	0.2

PCR was performed in a PTC-200 Thermal cycler (MJ Research, Watertown, MA). The PCR reaction program was run as follows: the reaction mixture was maintained at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 56°C annealing temperature for 45 s, and 72°C for 45 s. The reaction was then maintained at 72°C for 10 min, and then held at 4°C until reactions were retrieved from the thermal cycler. The IS-RS-5′ and IS-NS-3′ primers were tested separately by the protocol described by Dombrowski et al. (2006), in which a Qiagen kit was used to make up the reaction volumes (Dombrowski et al., 2006).

The eight different primer pairs were then tested on the DNA of the eight different contaminant fungi (Section 4.2.2) as well as the E^- plant DNA. This was to check whether the product length differed from that of the *Neotyphodium* endophytes. RT-qPCR measures the amplification of all products in the reaction volume. Therefore, a primer pair that would not generate any product when tested on contaminant DNA would be required for the development of the RT-qPCR protocol.

4.2.4. Gel electrophoresis

Products were separated on 2% agarose gels (1 g agarose/50 ml 1 × TAE buffer). The gels were run at 100 volts for approximately 1 h. Gels were visualised by staining with 0.5 µg/ml ethidium bromide. Gels were illuminated and photographed using a Chemi-genius^2 Gel Imaging System (Syngene).

4.2.5. Primer testing using real-time PCR

The primer pairs that were observed to amplify a single *Neotyphodium* product (across all four strains) and not amplify any contaminant or plant DNA had their sensitivity tested using RT-qPCR. First, one of the primers pairs was tested on endophyte DNA diluted 1000-fold, 10,000-fold, and 100,000-fold its original concentration (0.5-1 µg µl^{-1}) to determine the detection limit of the RT-qPCR assay. E^+ plant DNA of each of the four endophyte-grass combinations was then diluted to 10-fold, 100-fold, and 1000-fold from its original concentration (0.5-1 µg µl^{-1}) to determine the detection limit of the RT-qPCR assay *in planta* and which of the primer pairs was the most specific to endophyte DNA. Real-time PCR reactions were made up in 0.2 ml 8-strip PCR tubes. The reaction volume was as follows: 15 µl of Faststart SYBR Green Master Mix (Roche Diagnostics); 12 µl of dH_2O; 1 µl of forward primer (10 pmol/µl); 1 µl of reverse primer (10 pmol/µl); and 1 µl DNA (0.1-1 µg/ul). Real-time PCR was performed in a Stratagene® Mx3000p real-time PCR machine. The real-time qPCR reaction program (Fig. 4.1) was run as follows: the reaction mixture was maintained at 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 30 s, 56^{o}C annealing temperature for 1 min, and 72^{o}C for 30 s (the fluorescence of the SYBR green dye was measured at this point every cycle). The dissociation curve of the product was then measured by the reaction mixture being held at 95^{o}C for 1 min, then 55^{o}C for 30 s, and then the fluorescence of the SYBR green dye measured as the temperature was gradually increased to 95^{o}C where the reaction was held for 30 s. After this the program was complete and the block left to cool to room temperature.

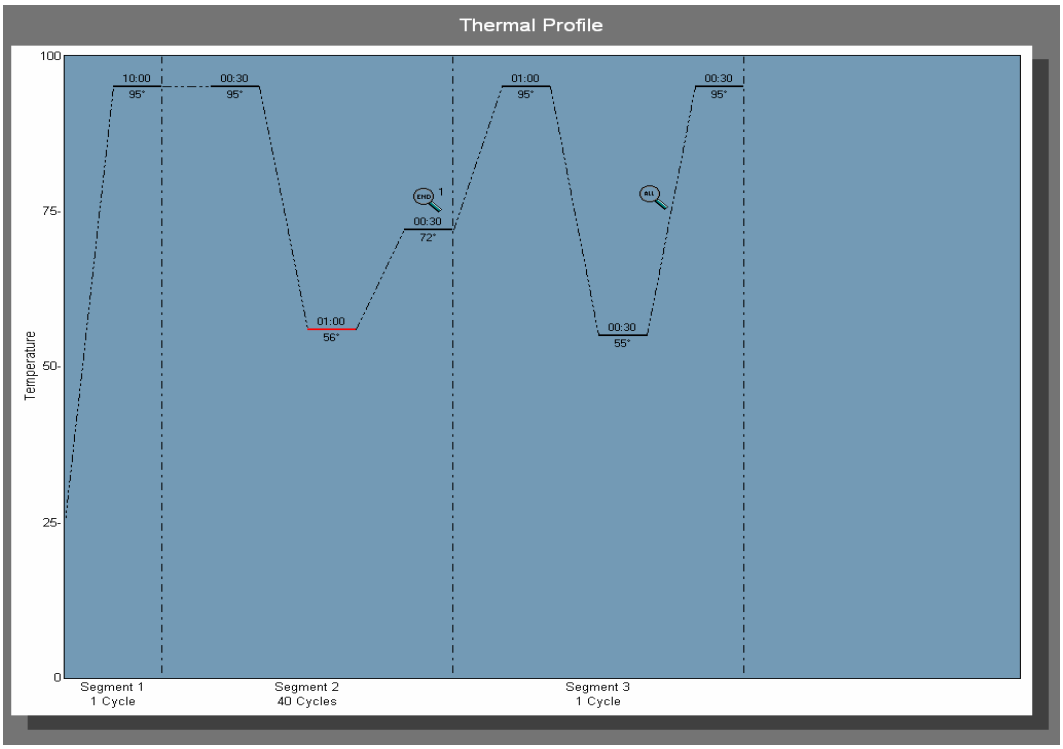


Figure 4.1. The RT-qPCR reaction program (segments 1 and 2) and melting curve (segment 3)

4.2.6. Protocol testing

To determine the age at which seedlings should be harvested for testing, groups of 50 FP 889 E^+ seedlings (Table 4.2) were germinated and grown for 5, 7, 10, 14 and 21 days in a growth room (25^{o}C, 24 h light). The above ground tissue was harvested and the DNA extracted via the modified CTAB method described in Section 4.2.2. The DNA (a combination of plant and endophyte) was then tested using RT-qPCR to determine at what age seedlings should be harvested.

For confirmation of the viable endophyte infection rate, three seed samples were taken from each of the four endophyte/host combinations (Table 4.2), germinated in a glasshouse and left to grow for four weeks. After this time 50 seedlings from each sample were examined via the histochemical staining method (Section 3.2.5). Three more samples were taken from each seed lot and sent for immunblot testing at AgResearch, Palmerston North.

Table 4.2. Host/endophyte combinations used for protocol testing

Endophyte strain	Endophyte species	Grass host species	Grass host cultivar
U2	*N. uncinatum*	*F. pratensis*	FP889
MaxP	*N. coenophialum*	*F. arundinacea*	Grasslanz Advance™
AR1	*N. lolii*	*L. perenne*	Revolution™
WT (wild type)	*N. lolli*	*L. perenne*	Matrix™

To test the hypothesis that endophyte DNA would amplify faster in a seed lot with a higher viable endophyte infection rate and to try and establish a standard curve relating amplification of endophyte DNA to the viable endophyte infection rate, E^+ and E^- seeds were mixed together to make E^+ proportions of 20, 40, 60, 80, 100%. Three samples were taken from each of these seed lots, germinated and grown in a growth room (24 h light, 25^{o}C) for 7 d. After this, the above ground tissue of 50 seedlings from each sample was harvested, weighed to obtain a fresh weight (FW), and the DNA extracted via the method described in Section 4.2.2. The DNA extracted from each sample was re-suspended in 90 µl of dH_2O so the variation in the amount of DNA extracted from each sample could be analysed. The concentration of extracted DNA was measured with a Nanodrop® spectrophotometer. Concentrations were then adjusted so that they were equal for RT-qPCR testing. Each sample was then tested using RT-qPCR; the RT-qPCR reaction volumes and the RT-PCR reaction program were the same as described in Section 4.2.5.

4.2.7. Graphical analysis

Graphical analysis of the FW, DNA concentration, and C_T values was done using Microsoft Excel®.

4.3. Results

4.3.1. Primer Design

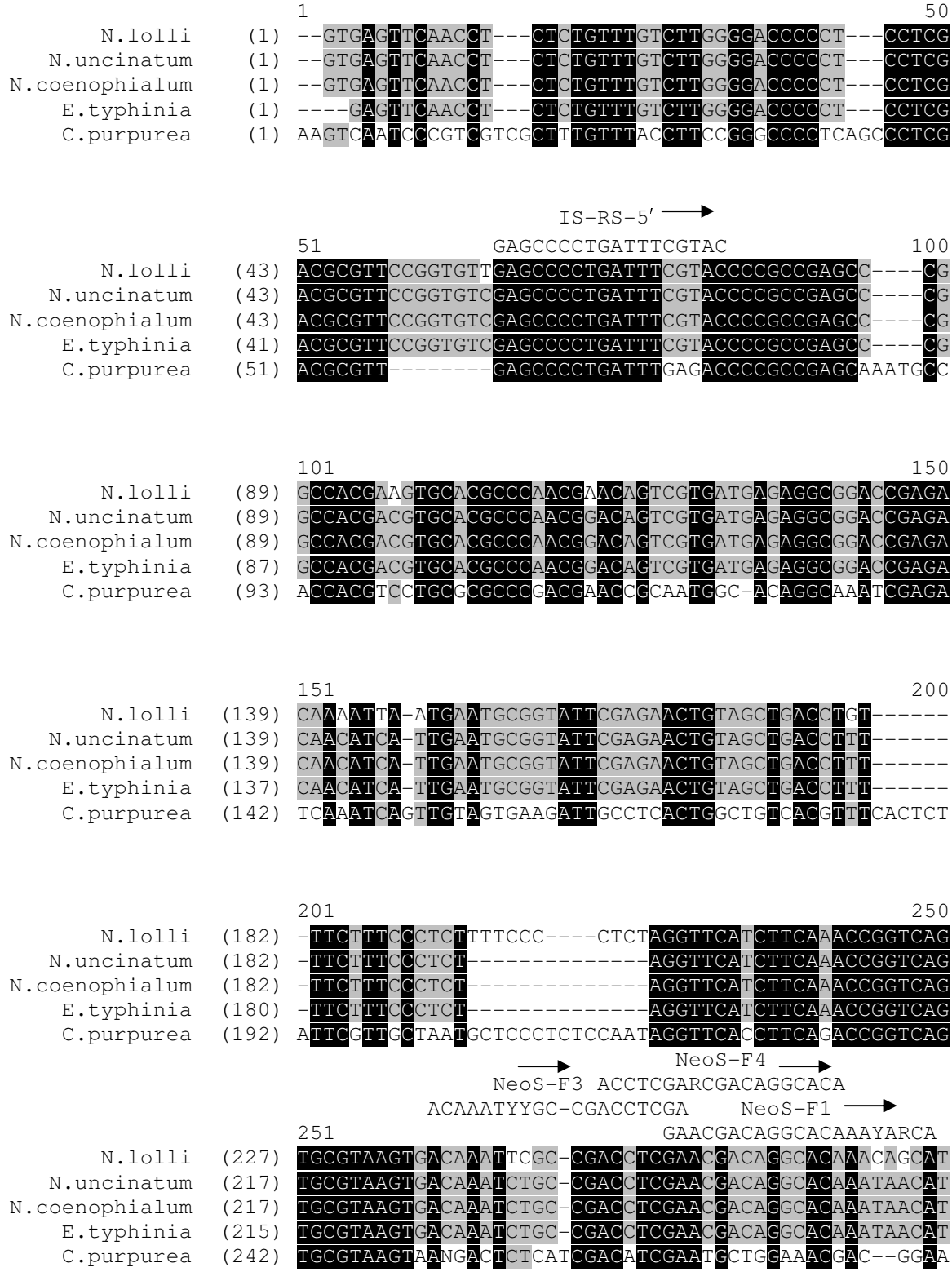
Searching of the GenBank® database revealed that there was more consistent genomic sequence for the *β*-tubulin gene (*tub2*) than there was for the ribosomal RNA internal transcribed spacer (*its1*, *its2*, *its3 and its4*) genes of the *Neotyphodium*/*Epichloë* genus. Thus, it was decided to design primers for *tub2*. Overall, there were over 100 genomic sequences of *tub2* for the *Neotyphodium*/*Epichloë* endophytes. However, only four of these were for *N. coenophialum*, two for *N. lolii* and one for *N. uncinatum*. Genomic sequence of *tub2* was limited for the common contaminant species (five sequences) and the host grasses (two sequences, both for *L. perenne*). However, sequences of *tub2* were available for the closely related contaminant species *C. purpurea*, *C. militaris* and *A. stricta* (four sequences in total).

The Genomic sequence of *tub2* for the *Neotyphodium*/*Epichloë* endophytes was not complete. Most sequences began just before or in the coding region of exon 1 and ended somewhere in exon 4 (Fig. 4.2). Several regions of introns 2 and 3 of *tub2* sequences were relatively conserved amongst *Neotyphodium*/*Epichloë* endophytes, but not between them and their close contaminant relatives (*C. purpurea*, *C. militaris* and *A. stricta*) (Fig. 4.2). This provided valuable points for the design of *Neotyphodium*/*Epichloë* specific primers. Based on these conserved regions, four forward primers were designed to be specific to intron 2 and two reverse primers were designed to be specific to intron 3 with the expected product being that of exon 3 (Fig. 4.2) (Table 4.3). These primers were 19-26 bases long, had melting temperatures (Tm) between 58.2-64.8^{o}C, resulting in eight different primer pairs generating PCR products of approximately 200 base pairs (bp) in length (Table 4.4). It was considered too difficult to design more than two reverse primers because of multiple adenine (A) and guanine (G) repeats in intron 3 of the *Neotyphodium tub2* gene resulting in undesirable melting temperatures (Tm) and/or primer dimers and/or potential hairpin formation (Fig. 4.2). The IS-RS-5′ and IS-NS-3′

primers described in Dombrowski et al. (2006) were found to have been designed to be specific to regions of exons 1 and 3 respectively of *tub2* where there was more conservation of the genomic sequence between the *Neotyphodium*/*Epichloë* endophytes and the contaminants (Fig. 4.2).

Table 4.3. *Neotyphodium* specific (NeoS) primer sequences and melting temperatures

Primer name	**Sequence (5′-3′)**	**Melting temperature (Tm) (^{o}C)**
NeoS-F1	GAACGACAGGCACAAAYARCA	61.8
NeoS-F2	AACTCACATTKMTTTGGGCAGG	60.9
NeoS-F3	ACAAATCYGCCGACCTCGA	63.4
NeoS-F4	ACCTCGARCGACAGGCACA	62.0
NeoS-R1	CGGTATGTAGCTCAATGGAGACT	58.2
NeoS-R2	GGTATGTATGTAGCTCAATGGAGACT	64.8
IS-RS-5′ (Dombrowski et al., 2006)	GAGCCCCTGATTTCGTAC	52.2
IS-NS-3′ (Dombrowski et al., 2006)	TTGAAGTAGACACTCATACGCTC	54.4


1 50
N.lolli (1) --GTGAGTTCAACCT---CTCTGTTTGTCTTGGGGACCCCCT---CCTCG
N.uncinatum (1) --GTGAGTTCAACCT---CTCTGTTTGTCTTGGGGACCCCCT---CCTCG
N.coenophialum (1) --GTGAGTTCAACCT---CTCTGTTTGTCTTGGGGACCCCCT---CCTCG
E.typhinia (1) ----GAGTTCAACCT---CTCTGTTTGTCTTGGGGACCCCCT---CCTCG
C.purpurea (1) AAGTCAATCCCGTCGTCGCTTTGTTTACCTTCCGGGCCCCTCAGCCCTCG
IS-RS-5′
51 GAGCCCCTGATTTCGTAC 100
N.lolli (43) ACGCGTTCCGGTGTTGAGCCCCTGATTTCGTACCCCGCCGAGCC----CG
N.uncinatum (43) ACGCGTTCCGGTGTCGAGCCCCTGATTTCGTACCCCGCCGAGCC----CG
N.coenophialum (43) ACGCGTTCCGGTGTCGAGCCCCTGATTTCGTACCCCGCCGAGCC----CG
E.typhinia (41) ACGCGTTCCGGTGTCGAGCCCCTGATTTCGTACCCCGCCGAGCC----CG
C.purpurea (51) ACGCGTT--------GAGCCCCTGATTTGAGACCCCGCCGAGCAAATGCC
101 150
N.lolli (89) GCCACGAAGTGCACGCCCAACGAACAGTCGTGATGAGAGGCGGACCGAGA
N.uncinatum (89) GCCACGACGTGCACGCCCAACGGACAGTCGTGATGAGAGGCGGACCGAGA
N.coenophialum (89) GCCACGACGTGCACGCCCAACGGACAGTCGTGATGAGAGGCGGACCGAGA
E.typhinia (87) GCCACGACGTGCACGCCCAACGGACAGTCGTGATGAGAGGCGGACCGAGA
C.purpurea (93) ACCACGTCCTGCGCGCCCGACGAACCGCAATGGC-ACAGGCAAATCGAGA
151 200
N.lolli (139) CAAAATTA-ATGAATGCGGTATTCGAGAACTGTAGCTGACCTGT------
N.uncinatum (139) CAACATCA-TTGAATGCGGTATTCGAGAACTGTAGCTGACCTTT------
N.coenophialum (139) CAACATCA-TTGAATGCGGTATTCGAGAACTGTAGCTGACCTTT------
E.typhinia (137) CAACATCA-TTGAATGCGGTATTCGAGAACTGTAGCTGACCTTT------
C.purpurea (142) TCAAATCAGTTGTAGTGAAGATTGCCTCACTGGCTGTCACGTTTCACTCT
201 250
N.lolli (182) -TTCTTTCCCTCTTTTCCC----CTCTAGGTTCATCTTCAAACCGGTCAG
N.uncinatum (182) -TTCTTTCCCTCT--------------AGGTTCATCTTCAAACCGGTCAG
N.coenophialum (182) -TTCTTTCCCTCT--------------AGGTTCATCTTCAAACCGGTCAG
E.typhinia (180) -TTCTTTCCCTCT--------------AGGTTCATCTTCAAACCGGTCAG
C.purpurea (192) ATTCGTTGCTAATGCTCCCTCTCCAATAGGTTCACCTTCAGACCGGTCAG
NeoS-F4
NeoS-F3 ACCTCGARCGACAGGCACA
ACAAATYYGC-CGACCTCGA NeoS-F1
251 GAACGACAGGCACAAAYARCA
N.lolli (227) TGCGTAAGTGACAAATTCGC-CGACCTCGAACGACAGGCACAAACAGCAT
N.uncinatum (217) TGCGTAAGTGACAAATCTGC-CGACCTCGAACGACAGGCACAAATAACAT
N.coenophialum (217) TGCGTAAGTGACAAATCTGC-CGACCTCGAACGACAGGCACAAATAACAT
E.typhinia (215) TGCGTAAGTGACAAATCTGC-CGACCTCGAACGACAGGCACAAATAACAT
C.purpurea (242) TGCGTAAGTAANGACTCTCATCGACATCGAATGCTGGAAACGAC--GGAA

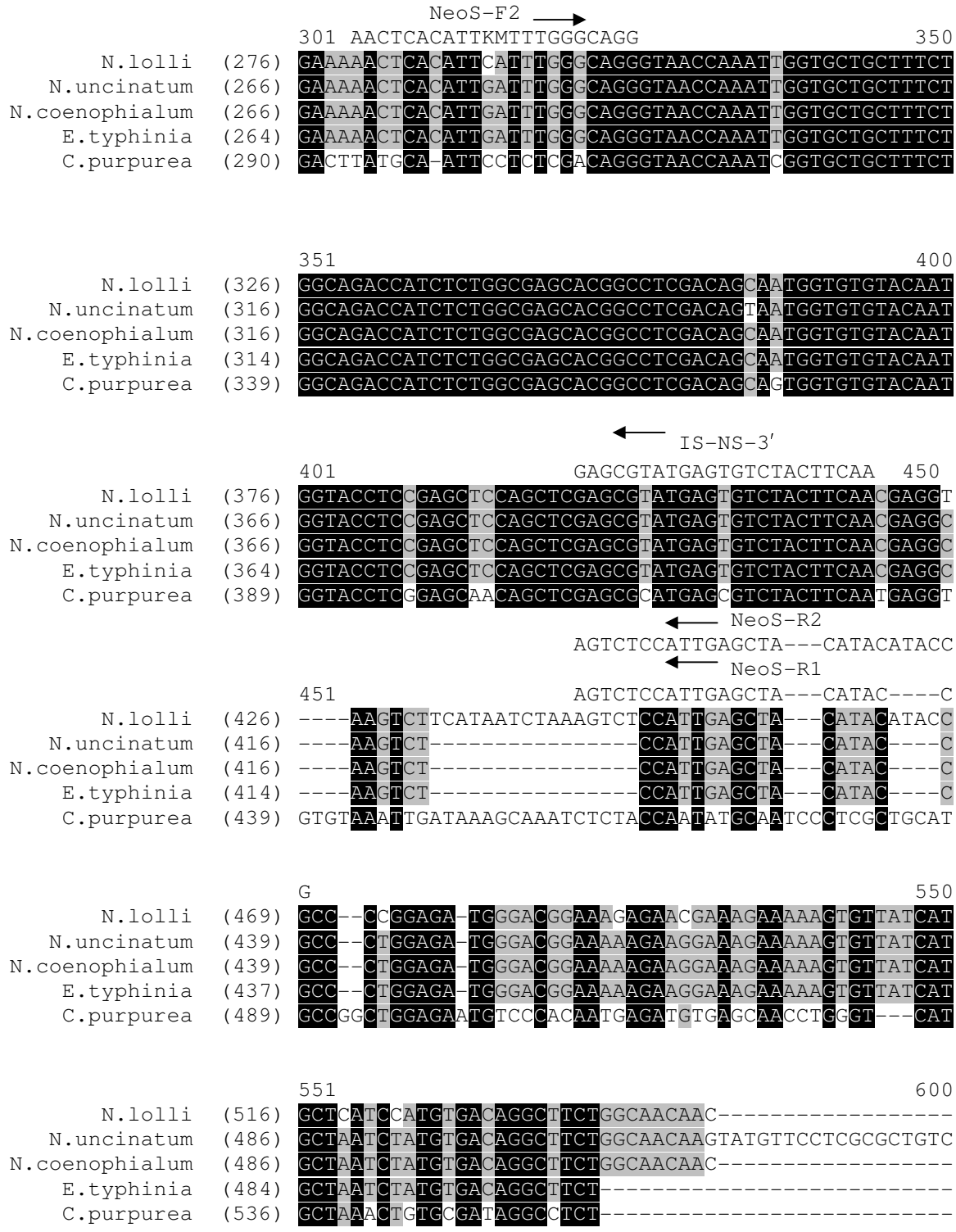


Fig 4.2. Clustal X alignment of the *β*-tubulin gene (*tub2*) for *E. typhinia* (GenBank® accession # AF062429), *N. coenophialum* (AY865627), *N. lolii* (AY865629), *N. uncinatum* (LO6946) and *C. purpurea* (AF062646) showing the positions of the primers shown in Table 4.3.

Table 4.4. Predicted product lengths of the nine different primer pairs for *N. uncinatum*, *N. coenophialum and N. lolii*.

Primer pair	**Predicted product length (base pairs)**		
	N. lolii	***N. uncinatum***	***N. coenophialum***
NeoS-F1 – NeoS-R1	214	198	198
NeoS-F2 – NeoS-R1	186	169	169
NeoS-F3 – NeoS-R1	231	215	215
NeoS-F4 – NeoS-R1	219	203	203
NeoS-F1 – NeoS-R2	213	197	197
NeoS-F2 – NeoS-R2	185	169	169
NeoS-F3 – NeoS-R2	230	214	214
NeoS-F4 – NeoS-R2	218	202	202
IS-RS-5′ – IS-NS-3′	370	358	358

4.3.2. Conventional PCR primer testing

All eight combinations of the designed *Neotyphodium* specific primers produced a single clear product of the predicted lengths (Table 4.4) when tested on all four strains of *Neotyphodium* endophytes (Fig. 4.3). Only the three species of *Alternaria* produced a product during contaminant testing with the different primer combinations (Fig. 4.4). However, the NeoS-F1 – NeoS-R2 and NeoS-F2 – NeoS-R2 primer pairs did not generate a product when tested on any of the *Alternaria* species making them safe for use in the development of the RT-qPCR protocol. The products that were generated by the other primer pairs when tested on the various *Alternaria* species were not similar in length to the *Neotyphodium* products (Fig. 4.4) suggesting that the amplified fragments were unlikely to be from the β-tubulin gene. Because the contaminant products were not similar in length to the *Neotyphodium* products all eight primer combinations could likely be used to accurately detect at least these three species of *Neotyphodium* endophytes *in*

planta. The IS-RS-5′ – IS-NS-3′ primer pair (Dombrowski et al., 2006) also generated products of the predicted length when tested on each of the four strains (Fig. 4.3).

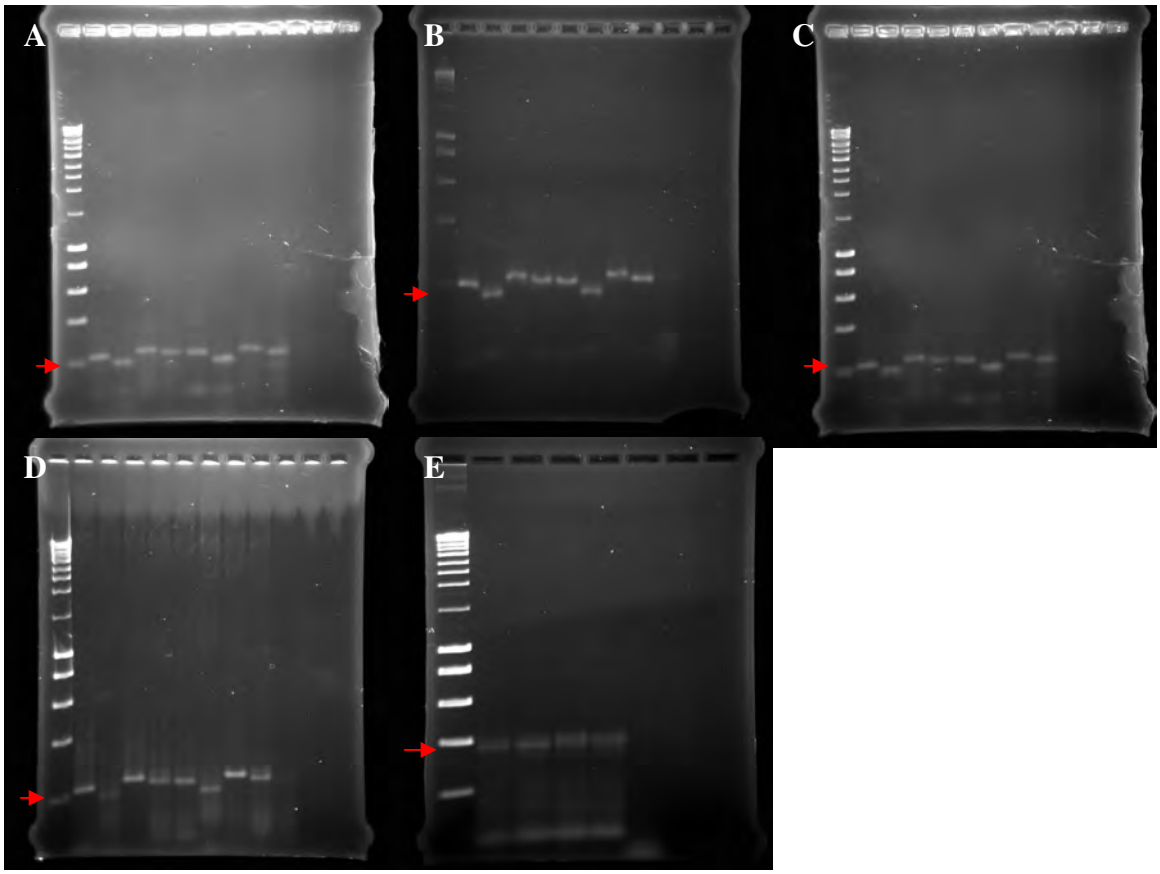


Figure 4.3. Primer combination testing on DNA extracted from the four endophyte strains; (A) U2, (B) MaxP, (C) AR1, and (D) WT. Lanes 1-10 (left to right) on each gel were as follows: 1, 1 Kb DNA ladder; 2, NeoS-F1-NeoS-R1; 3, F2-R1; 4, F3-R1; 5, F4-R1; 6, F1-R2; 7, F2-R2; 8, F3-R2, 9, F4-R2; 10, negative control (endophyte DNA replaced with H_2O, a random primer was chosen). Red arrows indicate 200 bp band of the 1 Kb DNA ladder. (E) Testing of the IS-RS-5′ – IS-NS-3′ primer pair (Dombrowski et al., 2006). Lanes 1-5 (left to right) were as follows: 1, U2; 2, MaxP; 3, AR1; 4, WT; 5, negative control. Red arrow indicates the 400 bp band of the 1 Kb ladder.

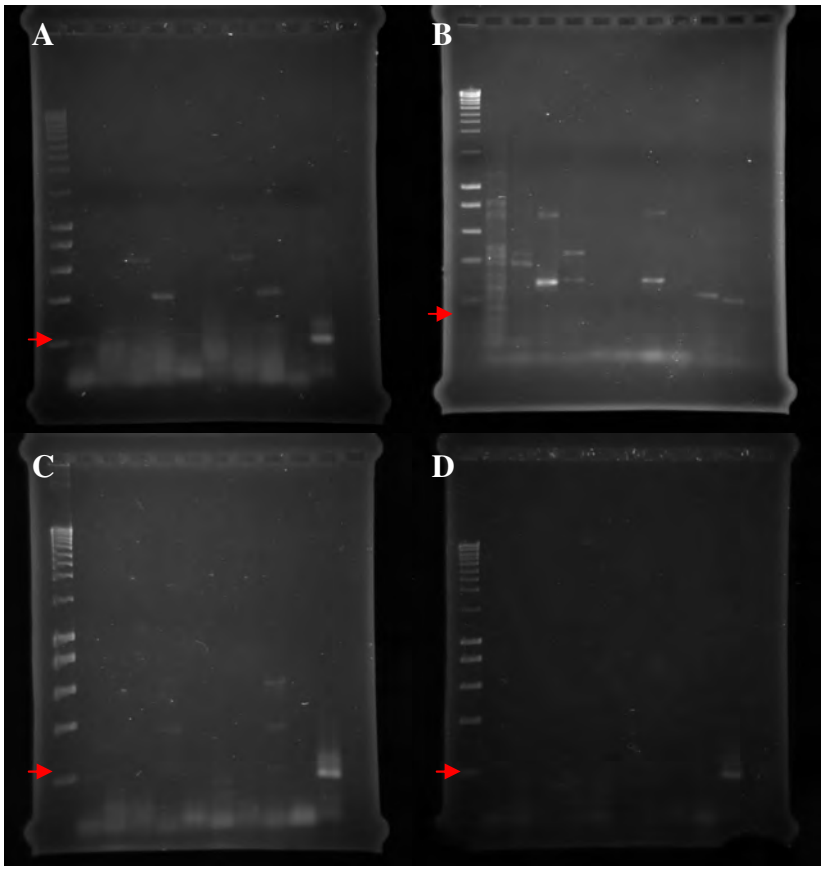


Figure 4.4. Primer combination testing on contaminant species DNA; (A) *Alternaria sp.1*, (B) *Alternaria sp.2*, (C) *Alternaria sp.3*, and (D) *C. purpurea*. Lanes 1-11 (left to right) on each gel were as follows: 1, 1 Kb DNA ladder; 2, NeoS-F1-NeoS-R1; 3, F2-R1; 4, F3-R1; 5, F4-R1; 6, F1-R2; 7, F2-R2; 8, F3-R2, 9, F4-R2; 10, negative control, 11, positive control (endophyte DNA) (the *Alternaria sp.2* gel has two positive controls (lanes 10-11), the negative control was lane 12). Gels for the other contaminant species DNA or the nil plant DNA were not shown as no products were generated by any primer pair (i.e. gels had the same appearance as the *C. purpurea* gel). Red arrows indicate 200 bp band of the 1 Kb DNA ladder.

4.3.3. Detection of *Neotyphodium* endophytes *in planta* using the NeoS-F2 – NeoS-R2 primer pair

The NeoS-F2 – NeoS-R2 primer pair demonstrated no difficulties in detecting *Neotyphodium* endophytes in E^+ seed or seedlings (Fig. 4.5 a, b). Throughout the course of this thesis it generated a clear product no matter what the concentration of E^+ plant

DNA (often the concentration was not even checked). The same could not be said for the IS-RS-5′ – IS-NS-3′ primer pair. It generated secondary products, or in some cases no product (data not shown), almost every time it was tested on E^+ plant DNA (Fig. 4.5 c). This made it unsuitable for use in the development of the RT-qPCR protocol.

The NeoS-F2 – NeoS-R2 primer pair also generated products of a different length depending on what endophyte strain it was tested on (Fig. 4.5) as was expected (Table 4.4). However, the *N. coenophialum* product was expected to be of the same length as the *N. uncinatum* product. Gel electrophoresis showed that the *N. coenophialum* product was more similar in length to the *N. lolii* product than to *N. uncinatum* (Fig. 4.5). This was not completely unexpected as one (accession # X56847) of the four *tub2* sequences for *N. coenophialum* in GenBank® was similar to that of the *N. lolii* sequences whereas the other three were more similar to the single *N. uncinatum tub2* sequence.

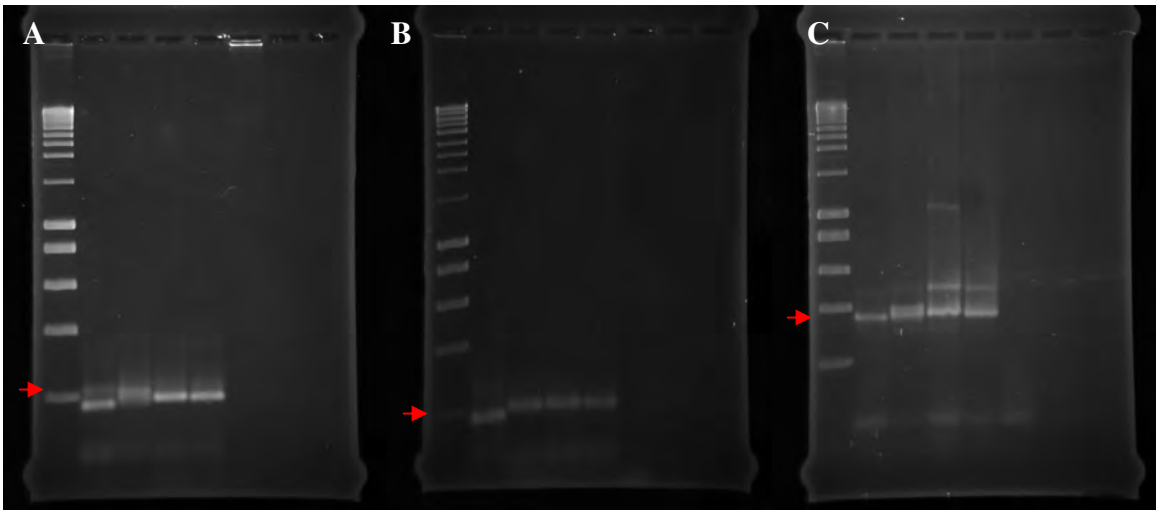


Figure 4.5. Detection of the four strains of *Neotyphodium* endophytes in (A) E^+ seedlings and (B) E^+ seed using the NeoS-F2 – NeoS-R2 primer pair. (C) Detection of the four strains of *Neotyphodium* endophytes in E^+ seedlings (using the same DNA as in (a)) using the IS-RS-5′ – IS-NS- 3′ primer pair. Lanes (1) 1 Kb DNA ladder, (2) U2, (3) MaxP, (4) AR1, (5) WT, (6) negative control (DNA replaced with H_2O). Red arrows indicate the 200 bp band (A, B) and 400 bp band (C) of the 1 Kb ladder.

4.3.4. RT-qPCR primer testing of the NeoS-F1 – NeoS-R2 and NeoS-F2 – NeoS-R2 primer pairs

The NeoS-F2 – NeoS-R2 primer pair was able to detect pure endophyte DNA down to a concentration of 0.005 ng μl^{-1} (lowest concentration tested) (Fig. 4.6). The amplification plot was beginning to loose its shape at the 0.005 ng μl^{-1} concentration suggesting that this concentration of endophyte DNA is the likely limit of the RT-qPCR assay to precisely quantify the endophyte DNA. A difference of approximately three threshold cycle values (C_T, cycle numbers at which a certain amount of amplified products were first detected) was observed between the serial dilutions of pure endophyte DNA (0.5, 0.05, and 0.005 ng μl^{-1}) (Fig. 4.6), with the detected concentration matching well with the dilution factors.

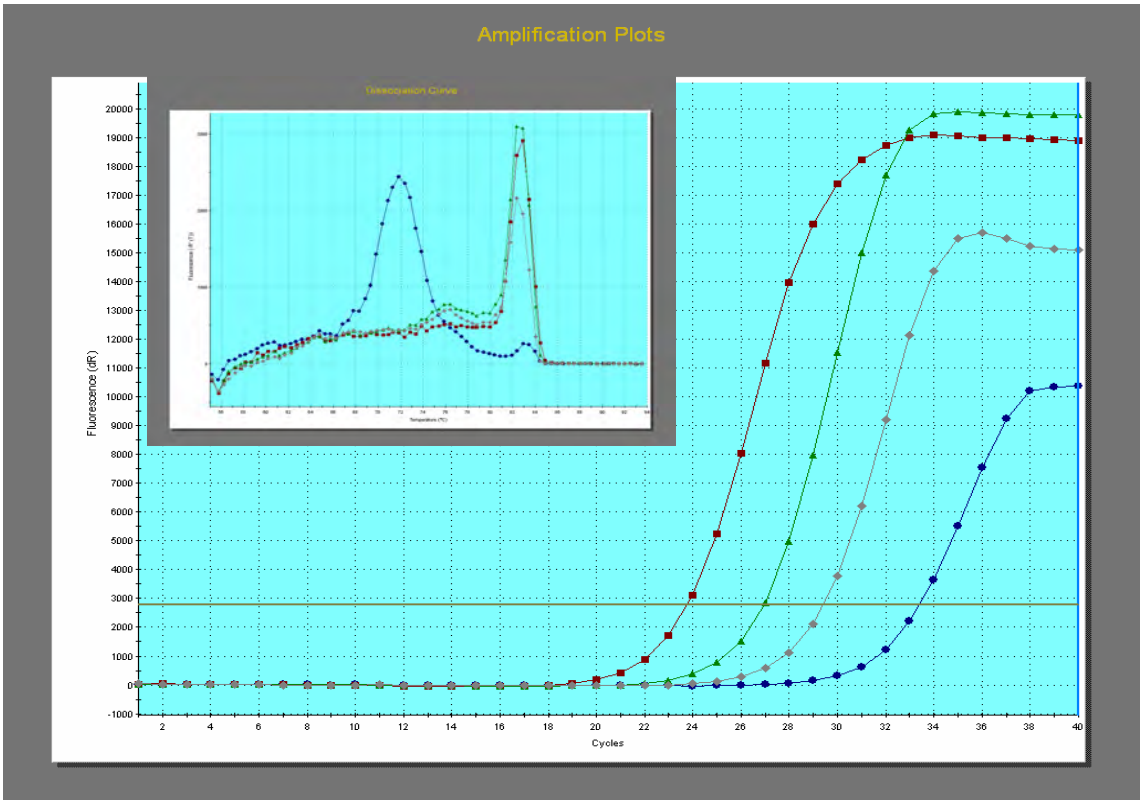


Figure 4.6. Amplification plots and dissociation curves of U2 DNA diluted to (red line) 0.5, (green line) 0.05, and (grey line) 0.005 ng μl^{-1} using the NeoS-F2 – NeoS-R2 primer pair. The negative control (DNA replaced with H_2O) is the blue line, the large peak in its dissociation curve represents primer dimers.

The NeoS-F2 – NeoS-R2 primer pair was shown to be the most specific to all four strains. It began to amplify the product faster than the NeoS-F1 – NeoS-R2 primer pair in 92% of the E^+ plant DNA concentrations tested across all four strains (Table 4.5). It always had the lowest C_T value (first to begin amplification of the product) at the 1000-fold dilution (0.5-1 ng μl^{-1} E^+ plant DNA) (Fig. 4.7). Being able to amplify a product at this concentration suggests that the NeoS-F2 – NeoS-R2 primer pair (and the NeoS-F1 – NeoS-R2 pair) would be able to detect one E^+ seedling in 1000 seedlings. Although not tested for their sensitivity using RT-qPCR, it is likely that the other six primer pairs would also have this degree of sensitivity. The difference between the C_T values of the serial dilutions of the E^+ plant DNA was not as uniform as it was with serial dilutions of pure endophyte DNA. The difference in the C_T values between concurrent serial dilution was always less than three and was variable (Table 4.5).

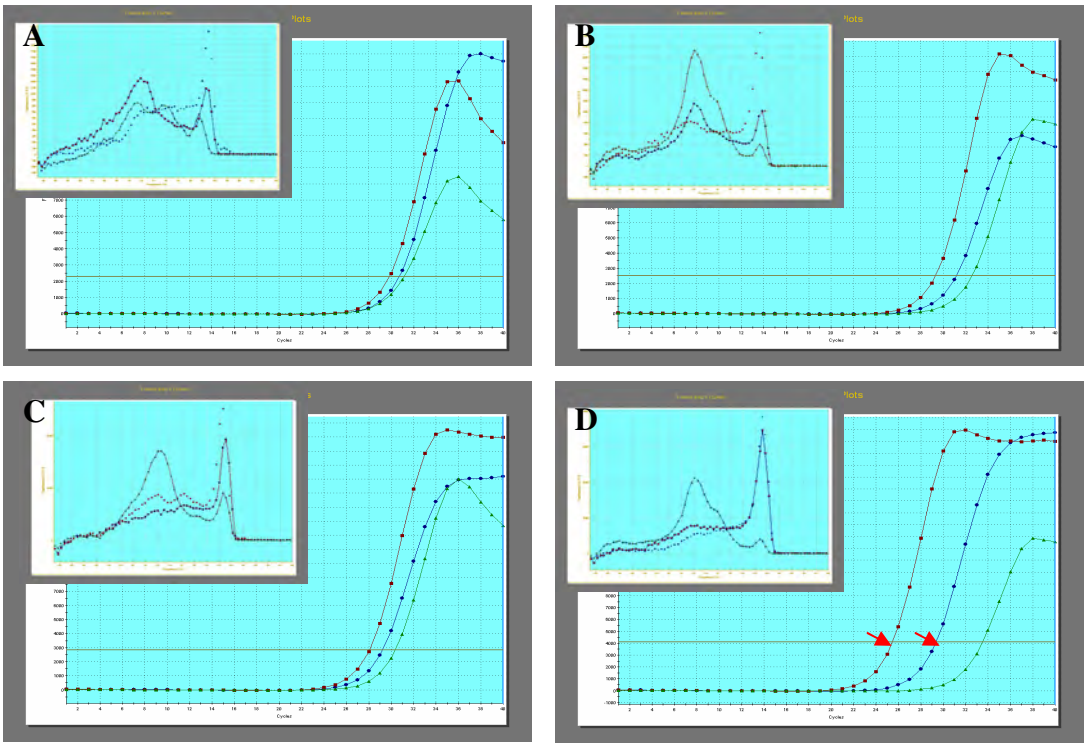


Figure 4.7. Amplification plots and dissociation curves of (A) U2, (B) MaxP, (C) AR1, and (D) WT E^+ plant DNA diluted to 0.5-1 ng μl^{-1} (one thousandth its original concentration). Amplification of the product using the NeoS-F2 – NeoS-R2 primer pair (red line) and the NeoS-F1 – NeoS-R2 primer pair (blue line). The negative control (DNA replaced with H_2O) is the green line.

Table 4.5. C_T values from the amplification plots of U2, MaxP, AR1, and WT E^+ plant DNA diluted 10-fold, 100-fold, and 1000-fold its original concentration (0.5-1 $\mu g\ \mu l^{-1}$) and tested with the NeoS-F1 – NeoS-R2 and NeoS-F2 – NeoS-R2 primer pairs. The C_T values represent the PCR cycle number at which the exponential phase of amplification of the product was reached (indicated by the red arrows in Fig. 4.7 d)

Strain	**U2**		**MaxP**		**AR1**		**WT**	
Primer pair	**F1-R2**	**F2-R2**	**F1-R2**	**F2-R2**	**F1-R2**	**F2-R2**	**F1-R2**	**F2-R2**
10-fold	26.46	26.22	27.01	26.57	27.12	25.56	25.42	22.42
100-fold	28.45	28.80	28.30	28.78	28.42	26.82	26.83	24.03
1000-fold	31.10	30.23	31.46	29.59	29.26	28.07	28.77	24.90

4.3.5. Determination of optimal seedling age

RT-qPCR testing of the DNA extracted from the above ground tissue of the FP889 E^+ seedlings of various ages (5, 7, 10, 14 and 21 d) revealed that the NeoS-F2 – NeoS-R2 primer pair could detect endophyte infection just five days after germination (DAG) (Fig. 4.8). However, seven DAG was decided upon as the optimal age for harvest because at this age all 50 seedlings were more uniform in size and, therefore, would yield a more uniform amount of DNA. There was little difference in the amplification efficiency of the NeoS-F2 – NeoS-R2 primer pair from seven DAG onwards (Fig. 4.8). Seven DAG was also decided upon for ease of planning the experiment and because it would work well if the protocol was developed into a commercial test (e.g. plant on a Monday, harvest on a Monday).

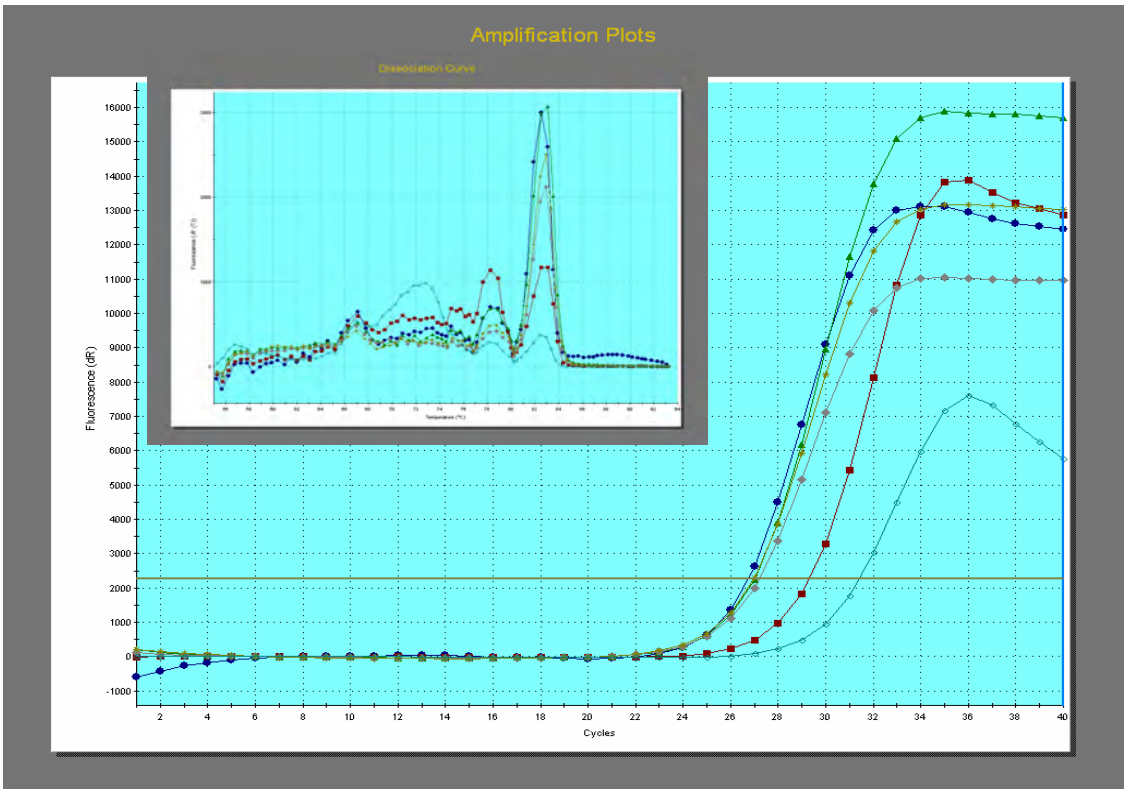


Fig 4.8. Amplification plots and dissociation curves of DNA extracted from 50 E^+ FP889 seedlings after 5 (red), 7 (green), 10 (grey), 14 (brown) and 21 (dark blue) DAG growth at 25°C. The negative control (DNA replaced with H_2O) is the light blue line.

4.3.6. Protocol testing

Only the FP889 and Revolution™ cultivars were used for the final stage in the development of the protocol. This was because the MaxP infection percentage of the Grasslands Advance™ cultivar was found to be low (63%) (Table 4.6). It was also observed to be unstable via repeated grow out tests. Therefore, the the percentage of E^+ seedlings for the Grasslands Advance™ cultivar required for the protocol was unable to be estimated accurately. The Matrix™ (infected with WT) was not included because WT and AR1 were of the same species (*N. lolii*) and it was anticipated that they would produce a similar result.

Histochemical staining and immunoblot testing of the four E^+ grass cultivars produced similar results in terms of the viable endophyte infection percentages of the FP889 (U2), Grasslands Advance™ (MaxP) and Revolution™ (AR1) seed lots (Table 4.6). However, the viable endophyte infection percentage of the Matrix™ (WT) seed lot was significantly higher when determined by histochemical staining (96.7%) compared to immunoblot test (87.7%). Only one sample of FP889 was tested via each method as seed for this line was very limited.

Table 4.6. Viable endophyte infection rates (% E^+ seedlings) of seed lot samples determined by histochemical staining and immunoblot testing.

Cultivar/endophyte (sample)	**% E^+ seedlings determined via histochemical staining**	**% E^+ seedlings determined via immunoblot testing**
Revolution/AR1 (1)	90	94.6
Revolution/AR1 (2)	98	97.8
Revolution/AR1 (3)	100	97.8
Revolution/AR1 (average)	**96**	**96.7**
G. Advance/MaxP (1)	64	62.1
G. Advance/MaxP (2)	70	65.3
G. Advance/MaxP (3)	54	64.1
G. Advance/MaxP (average)	**63**	**63.8**
Matrix/WT (1)	98	85.7
Matrix/WT (2)	96	88.2
Matrix/WT (3)	96	89.2
Matrix/WT (average)	**96.7**	**87.7**
FP889/U2	**100**	**100**

Fresh weights of the above ground tissue harvested from FP889 seedlings germinated from seed lots with known E^+ plant percentages of 0, 20, 40, 60, 80 and 100% were comparable to each other suggesting that there should be little difference in the amount of plant DNA extracted from each replicate; the only difference should be in the amount of

endophyte DNA (Fig. 4.9). The same was true for the fresh weights of the Revolution™ seedlings (Fig. 4.9). However, the amount of DNA extracted from each replicate varied four-fold even though care was taken (using a timer) to make sure that there was no difference in the modified CTAB method used for DNA extraction between each replicate. Although no statistical analysis was conducted, the results do suggest that significantly more DNA was extracted from 100% E^+ FP889 seedlings and the 80 and 100% E^+ Revolution™ seedlings compared to the other seedlings from each cultivar (Fig. 4.9 b).

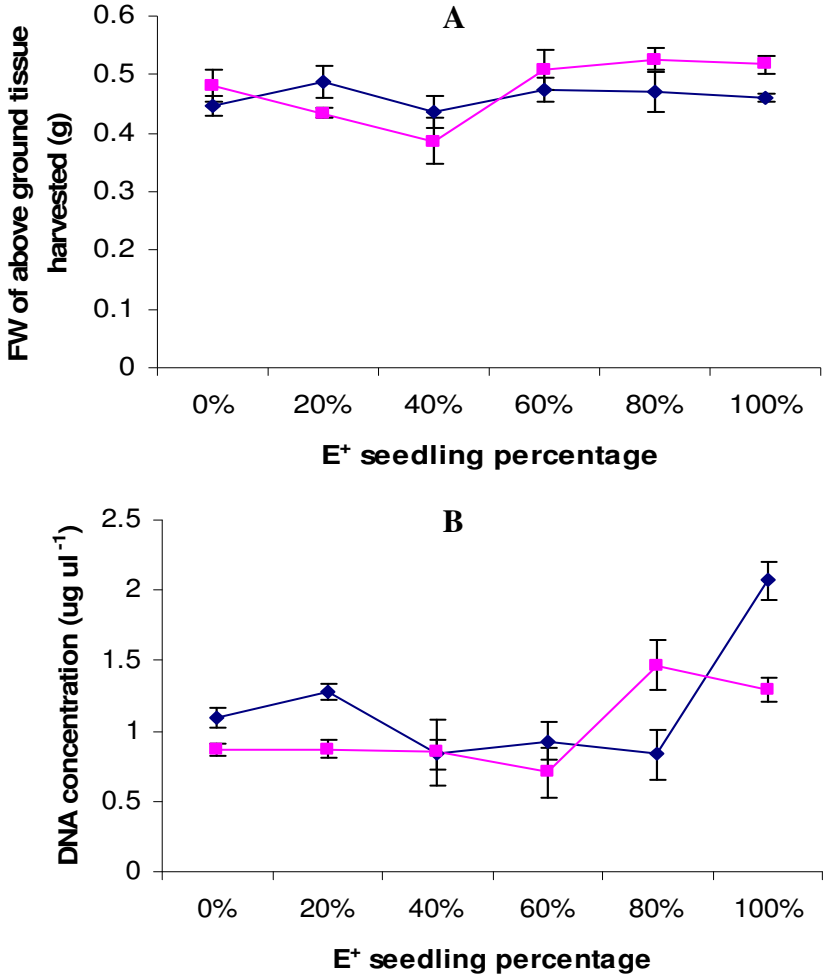


Figure 4.9. (A) Fresh weights of above ground tissue and (B) the concentration of DNA extracted compared to the percentage of E^+ seedlings in each sample of FP889 (blue line) and Revolution™ (pink line). Means (n=3) are indicated with error bars equal to ± 1 S.E.

Relating the amount of endophyte DNA to the known percentage of E^+ plants in a seed lot was unsuccessful for both FP889 and Revolution™. There appeared to be no trend between the C_T values and the percentage of E^+ seedlings in the samples as had been hoped for (Fig. 4.10). The only significant result was that of the 20% E^+ Revolution™ seedlings had a higher C_T value than the 40, 60, 80, and 100% E^+ seedlings (Fig. 4.11 b). The difference between the highest and lowest C_T values for the FP889 and Revolution™ seed lots were 3.37 and 3.09 PCR cycles respectively (Appendix 3). More endophyte DNA was often quantified (had a lower C_T value) from the lower percentage E^+ seedling replicates than the 100% E^+ seedlings. The dissociation curves also indicate that there may have been one or two E^+ seedlings in some of the 0% E^+ seedling replicates (Appendix 3). This supports earlier testing (Section 4.3.4) which suggested that the NeoS-F2 – NeoS-R2 primer pair would be sensitive enough to detect 1% endophyte infection of a seed lot.

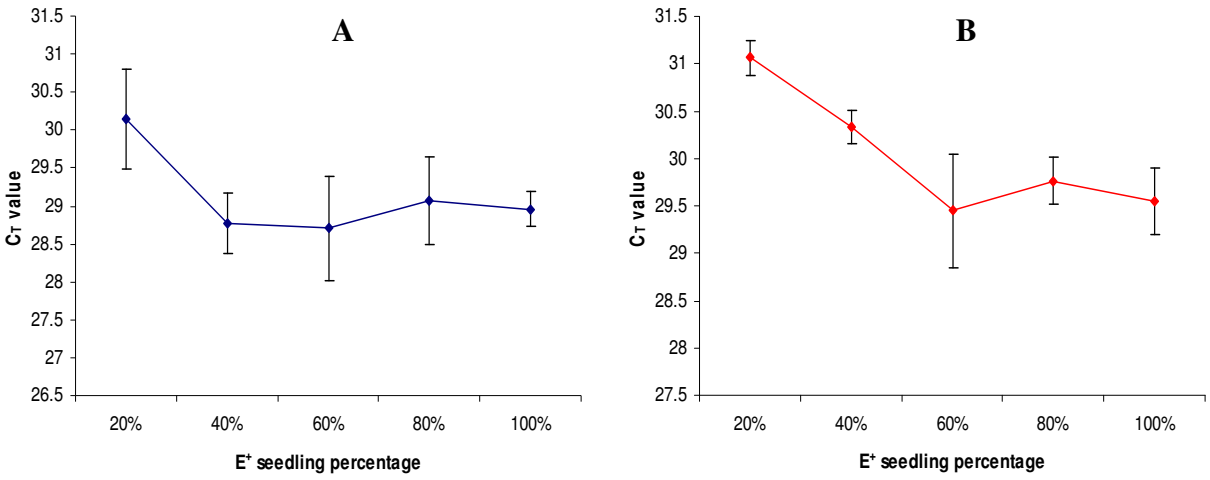


Figure 4.10. C_T values vs the known E^+ seedling percentage for (A) FP889 and (B) Revolution™. Means (n=3) are indicated with error bars equal to ± 1 S.E.

4.4 Discussion

4.4.1. The design of *Neotyphodium* specific primers

Dombrowski et al. (2006) reported that the IS-RS-5′ and IS-NS-3′ primers were specific to regions of the β-tubulin gene (*tub2*) of *Neotyphodium* species isolated from tall fescue, perennial ryegass and Italian ryegrass (Dombrowski et al., 2006) suggesting that primers could be designed to be specific to *tub2* of *Neotyphodium*/*Epichloë* endophytes. Doss et al. (1995) and Doss et al. (1998) also designed primers specific to *tub2* of *N. coenophialum* and *E. typhinia* (Section 1.6). However, these were never claimed to be specific to *tub2* of other *Neotyphodium*/*Epichloë* species nor was it claimed that they would not detect contaminant fungi (Doss and Welty, 1995; Doss et al., 1998).

In agreement with Dombrowski et al. (2006), the eight primer pairs designed in this thesis amplified a single product from four strains of *Neotyphodium* endophytes and did not amplify any products of a similar length from common contaminant fungi found in/on grass seed/seedlings. The fact that all eight primer pairs generated a single clear product when tested on all four strains of endophyte suggests that there may be some discrepancies in the sequence data stored in Genbank®. The NeoS-R2 primer would not be expected to anneal with such high specificity to intron 3 of *tub2* of *N. uncinatum* nor the NeoS-R1 primer to intron 3 of *tub2* of *N. coenophialum* and *N. lolii* (Fig. 4.2). Percent similarities of *tub2* sequences were not determined in this thesis because of doubt over the sequence data from GenBank®.

The primers designed in this thesis are likely to be even more specific to *N. coenophialum*, *N.lolii* and *N.uncinatum* than the IS-RS-5′ - IS-NS-3′ primer pair designed by Dombrowski et al. (2006). This is because the NeoS- primers designed in this thesis were designed to anneal to the introns of *tub2*, where there was less conservation of sequence between the *Neotyphodium*/*Epichloë* endophytes and common contaminant fungi of grass seed/seedlings, whereas the IS-RS-5′ and IS-NS-3′ primers (Dombrowski

et al., 2006) were designed to anneal to the exons of *tub2* where there was more conservation of sequence between the *Neotyphodium*/*Epichloë* endophytes and the common contaminant species. Although Dombrowski et al. (2006) tested their primers on common contaminant DNA including that of *C. purpurea* it is not inconceivable that the IS-RS-5′ – IS-NS-3′ primer pair could generate a product similar in length to the *Neotyphodium* product if *C. purpurea* DNA was present in the reaction; especially when the fact that the NeoS-R1 and NeoS-R2 primers were able to anneal where they did is considered. Because *C. purpurea* is difficult to culture on agar it is recommended that a primer pair specific to it be designed for use as positive control in future studies to ensure that designed *Neotyphodium* specific primers have been tested on *C. purpurea* DNA.

Although it worked well in this thesis, *tub2* is not necessarily the best target for designing primers for the detection of *Neotyphodium* endophytes *in planta*. Many recent studies have focused on identifying and sequencing the genes involved in alkaloid biosynthesis (Panaccione et al., 2003; Wang et al., 2004; Spiering et al., 2005; Young et al., 2005; Young et al., 2006; Fleetwod et al., 2007; Schardl et al., 2007; Scott et al., 2007). Some of these genes are likely to be unique to *Neotyphodium* endophytes whereas common contaminant fungi and grass hosts are highly unlikely to have them. This makes them safe targets for primer design as there would be little risk that common contaminants or the grass host could generate a false positive. At the 6th International Symposium on Fungal Endophytes in Grasses (Christchurch, March, 2007) it was announced that the entire genome of *E. festucae* would be released later this year (Hesse et al., 2007). This will be a very useful tool for the design of primers specific to different genes of *Neotyphodium* endophytes.

4.4.2. The detection of *Neotyphodium* endophytes *in planta* using *Neotyphodium* specific primers

Although all were not tested, all eight primer pairs designed in this thesis are likely to be capable of detecting strains of *N. lolii*, *N. coenphialum* and *N. uncinatum in planta*. This is also the first time that the detection of *N. uncinatum in planta* using *Neotyphodium*

specific primers has been reported. The primers designed in this thesis would likely detect a much greater range of *Neotyphodium* and *Epichloë* endophyte species than the three tested in this thesis.

The NeoS-F2 – NeoS-R2 primer pair designed and used in this thesis was more practical for the commercial detection of *Neotyphodium* endophytes *in planta* than the IS-RS-5′ – IS-NS-3′ primer pair designed by Dombrowski et al. (2006). The IS-RS-5′ – IS-NS-3′ primer pair often generated secondary products when tested on E^+ plant DNA (Fig. 4.5). Dombrowski et al. (2006) mentioned that the IS-RS-5′ – IS-NS-3′ primer pair generated secondary products at high concentrations of E^+ DNA although they did not present images of entire gels, just the narrow region where the desired product had migrated to. Dombrowski et al. (2006) also stated that checking and adjusting the concentration of E^+ plant DNA was critical for the detection of *Neotyphodium* endophytes *in planta*. However, this was not the case for the NeoS-F2 – NeoS-R2 primer pair where a single clear product was generated at all concentrations of E^+ plant DNA tested (Fig. 4.5). When using the NeoS-F2 – NeoS-R2 primer pair for the detection of *Neotyphodium* endophytes *in planta* there appeared to be no need to dilute or even check the concentration of the E^+ seedling or E^+ seed DNA providing the DNA filament was re-suspended in an appropriate amount of distilled water.

The difference in the product length generated by NeoS-F2 – NeoS-R2 primer pair between the strains of *N. uncinatum* and *N. lolii*/*N. coenophialum* has also been shown to be a useful tool in controlled commercial plant breeding programs. At present, Cropmark Seeds Ltd has only two strains of *Neotyphodium* endophytes (U2 and AR1) intensively involved in their plant breeding program. Occasionally, a plant breeder can become unsure as to which species of endophyte is present in a grass plant. A PCR check with the NeoS-F2 – NeoS-R2 primer pair could be used as a cheap and rapid method to determine which species of endophyte is present in a grass plant. The only other ways to determine the species of the endophyte would be to either isolate the endophyte from a

tiller and determine which species it is from its appearance *in vitro* (time consuming) or get an alkaloid profile conducted on some of the leaf material (costly, $100 per profile). However, the range of endophyte species and strains in the Cropmark Seeds Ltd plant breeding program is set to increase significantly in coming years. The design of primer pairs specific to individual strains or primer pairs that generate products of different lengths when tested on different strains is seen as highly desirable for plant breeding purposes (pers. comm. Nick Cameron, Cropmark Seeds Ltd.). It is unlikely that this would be able to be achieved using the *tub2* gene because it is relatively conserved between strains and species of *Neotyphodium*/*Epichloë* endophytes. The release of the *E. festucae* genome (Hesse et al., 2007) will provide a stepping stone for the possible development of such primers.

It also must be noted that RT-qPCR is more sensitive for the detection of *Neotyphodium* endophytes *in planta* compared to conventional PCR in combination with gel electrophoresis. It was noticed during the course of this thesis that small peaks (<1000 fluorescence) shown on the dissociation curve were not clearly visible when the products of the RT-qPCR reactions were run on a gel (data not shown). It is unlikely that the product of a conventional 35 cycle PCR reaction using one of the primer pairs designed in this thesis to detect endophyte DNA in E^+ plant DNA diluted to 0.5-1 ng μl^{-1} would be clearly visible on a gel, whereas the peak would be visible on the dissociation curve generated by the RT-qPCR machine.

During the development of the RT-qPCR protocol small peaks (200-400 fluorescence) at the same Tm as the *Neotyphodium* product were observed in the negative control (e.g. Fig. 4.6, blue line). A band was never observed when the negative control of a conventional PCR reaction was run on a gel unless there was obvious contamination of the reaction volume. The source of these peaks remains unknown (it is unlikely that every RT-qPCR negative control was contaminated) but they would be unlikely to cause a falsely positive result because they were always much smaller than a peak that was generated when even a small amount of endophyte DNA was present in the reaction. In the case of Figure 4.6, the C_T value of the negative control was higher than that of the sample with lowest

endophyte DNA (0.005 ng μl^{-1} , the grey line) by four cycles. Therefore, the concentration of its initial template would be about 100-fold lower than the latter. Furthermore, its peak area in the dissociation curve plot was about 0.025-fold of the big peak produced by the primer dimer. Considering that the ratio between the peak areas in the dissociation curves is more or less proportional to the concentrations of the PCR products (Ruiz-Ponte et al., 2000), the actual concentration of the template generating this small peak would be 3000-fold lower than that of the sample containing 0.005 ng μl^{-1} endophyte DNA. Such a small amount of DNA would be negligible for endophyte DNA quantification. E^- plant DNA produced a very similar sized size peak to that of the negative RT-qPCR control (data not shown). RT-qPCR testing of the IS-RS-5′ - IS-NS-3′ primer pair revealed that they too produced a small peak (at the same Tm as the *Neotyphodium* product) in the negative control (data not shown).

4.4.3. Determining the viable endophyte infection rate of a seed lot

Determining the viable endophyte infection rate of a seed lot is a process that involves significant sources of error, and much of this will be hard to avoid if the method is to remain efficient for commercial use. The first source of error when using histochemical staining or the immunoblot test to determine the endophyte infection rate of seed lot is that 50-100 seeds are often being used as a representative sample of several tonnes of seed. The second source of error when using histochemical staining as the detection method results from the examiner either not identifying the endophyte because of weak infection or poor staining technique or falsely identifying something else as endophyte and further that only a portion of the plant tissue can be visualised on a slide. The second source of error when using the imunnoblot test is that it can generate false negatives when there is weak infection or false positives from cross reactions with plant or contaminant fungal proteins. The results from this thesis, past literature (Barker et al., 2005; Hill et al., 2005; Dombrowski et al., 2006), and personal communications with people involved in the commercial determination of the viable endophyte infection rate of seed lots (Nick Grbavac, Agriquality; Wayne Simpson, Agresearch; and Nick Cameron, Cropmark Seeds

Ltd.) suggest that the second sources of error for the histochemical staining and immunoblot methods of testing are ±5% and ±10% respectively.

The major problem in developing a RT-qPCR protocol for determining the viable endophyte infection rate of a seed lot is that there are many more sources of error which are unable to be avoided before the final measurement (the C_T value of the seed lot) is made. Every time a supernatant is removed to a new tube, or an extraction is performed (e.g. with CTAB buffer or chloroform) during DNA extraction from seedlings there is a source of error. The uneven differences between the C_T values of the serial dilutions of E^+ plant DNA (Table 4.5) also suggest that every time a solution of E^+ plant DNA is pipetted there is another source of error. The serial dilution curve of the endophyte DNA (Fig. 4.6) suggests that the maximum difference there should be between the C_T values of a 20% E^+ and 100% E^+ seed lot is one and a half PCR cycles because the difference between the E^+ percentages of the seed lots represents a five fold dilution. When establishing a standard curve, relating the C_T value to the E^+ plant percentage of a seed lot, a small scale (a difference of 1.5 C_T values) on the Y axis is being related to a large scale (a difference of 80% in the E^+ plant percentage) on the X axis. Therefore, a small difference on the Y axis (the C_T value) will correspond to a large difference on the X axis (the E^+ plant percentage). Because of all of the sources of error in the preparation of a sample (which are additive), the final C_T value actually represents a range of C_T values and thus an even larger range of E^+ plant percentages. In this thesis, the range of C_T values obtained from seed lots with the same known E^+ plant percentage was large (up to 2.37 PCR cycles) (Appendix 3). The means of the C_T values calculated from the three replicates of seedlings with known E^+ plant percentages were also not significantly different from each other except in one case (Fig. 4.11). This resulted in the standard curve being relatively flat and not showing any significant trend. Therefore, it was not possible to predict the viable endophyte infection rate of a seed lot from its C_T value determined using RT-qPCR.

The variation in the endophyte infection rate of individual seedlings was also taken into account as a source of error when establishing the RT-qPCR protocol. It is one of the

major sources of error involved with the histochemical staining or immunoblots tests (i.e. weak infection generating a false negative). However, this source of error is likely to be reduced when using the RT-qPCR protocol as the testing method because of its high sensitivity and the fact that each sample consists of 50 seedlings rather than one.

In theory, it should be possible to develop a protocol for determining the viable endophyte infection rate of a seed lot using RT-qPCR. It was shown qualitatively, using the NeoS-F2 – NeoS-R2 primer pair, that there was viable endophyte in a 1% E^+ seed lot. However, quantitatively, only once was a difference determined between seed lots varying in their E^+ percentages by 20% (Fig 4.10 b). Based on the results in this thesis, it may be practically impossible to develop a commercial RT-qPCR protocol for determining the viable endophyte infection rate in seed lots of perennial ryegrass and meadow fescue.

Three replicates of the E^+ plant percentages (0, 20, 40, 60, 80 and 100%) were unable to give even a weak indication as to whether a standard curve of the desired nature, relating the C_T values of the E^+ seedling replicates to their viable endophyte infection rate, could be established. The ranges in the C_T values at each E^+ percentage (Appendix 3) suggest that increasing the number of replicates would be unlikely to produce a much stronger trend. This could also turn out to be a rather costly exercise as 15% of the FastStart SYBR Green Master Mix kit (Roche Diagnostics) was used every time a protocol test (Appendix 3) was run in this thesis (19 RT-qPCR reactions in total).

The recommended way of increasing the accuracy of the protocol and to try and develop a standard curve of the desired nature based on the results of this thesis would be to investigate ways of minimising the sources of error before the reactions are set up and then increasing the number of replicates if promising results are obtained. The modified CTAB method was occasionally observed to be unreliable during the course of this thesis. For example, two samples of plant material from the same plant of the same size were observed to yield totally different amounts of DNA (far greater than the differences in yield depicted in Fig. 4.9) even when the DNA extraction protocol was adhered to

rigorously. For this reason, trying other methods of DNA extraction is recommended. Using a crude DNA extract (e.g. the supernatant removed to a new tube in the third step of the modified CTAB method described in Section 4.2.2) rather than a purified one may also be a way of minimising the sources of error.

Chapter Five

Final discussion, recommendations and conclusions

Neotyphodium endophytes form mutalistic associations with many forage grasses throughout the world. The endophyte gains a place to live while the grass host gains increased resistance to herbivores and, in some associations, drought from the various alkaloids produced by the endophyte. *F. arundinacea* (tall fescue), *L. perenne* (perennial ryegrass) and *F. pratensis* (meadow fescue) are the most agronomically important cool season forage grasses in countries such as New Zealand, Australia, and the U.S.A., where large amounts of developed pastoral based agriculture takes place. As a consequence the endophyte species native to these grass species (*N. coenophialum*, *N. lolii* and *N. uncinatum* respectively) have become the most agronomically important endophyte species. Several strains of *Neotyphodium* endophytes (AR1, MaxQ, MaxP and AR37), referred to as novel strains, because they do not produce the anti-mammalian alkaloids but do still produce the alkaloids which confer insect and drought resistance to the host, have emerged on the commercial market in the past decade. Novel endophytes are very valuable to their seed company owners. CropMark Seeds Ltd. (the company supplying the majority of the funding for this research) owns the patent to the U2 strain of *N. uncinatum* (a novel endophyte) which they hope to commercialise in the near future by establishing associations between it and some of their forage grass varieties.

In this thesis, four of the aims were associated with optimising the methods used in the commercial development of grass cultivars with highly desirable agronomic characteristics infected with a novel endophyte. These aims included increasing the growth rate of strains *in vitro*, determining viability *in vitro*, optimising inoculation success, and establishing a more rapid method for determining endophyte viability in seed lots.

The results from this thesis show that the medium a *Neotyphodium* endophyte is cultured on can affect the morphology of the mycelium, the overall appearance of the colony and the radial growth rate of the colony. However, the degree or nature of the effect the medium has on the colony parameters was ultimately determined by the genotype of the strain. Several recommendations can be made for the three of the *Neotyphodium* endophyte strains involved in this thesis in terms of which type and concentration of medium is best for achieving the fastest rates of radial growth while maintaining a colony appearance suitable for inoculation and DNA extraction procedures. The U2 strain of *N. uncinatum* performed best on 1% (w/v) agar MEA and PDA media where it formed flatter faster radially expanding colonies than it did on the other media. The MaxP strain of *N. coenophialum* performed best on the 2% (w/v) MEA or 1% (w/v) agar PDA media. The MaxP colonies on these media were fast growing while at the same time producing a thicker drier mycelial mat in their outer regions compared to the thin or slimy-like mycelial mats in the outer regions of the colonies grown on the other media. The WT strain of *N. lolii* performed almost equally as well on four of the media (the 1% and 1.5% (w/v) agar MEA and PDA). However, the 1.5% (w/v) agar PDA generated the thickest, fluffiest colonies so it is the one recommended; 1.5% (w/v) agar MEA would suffice if PDA was not available. However, because of the overriding influence of genotype, no general recommendations can be made: the effect of different media on different strains would need to be determined strain by strain.

It is unlikely that artificial associations between *Neotyphodium* endophytes and grass hosts would be able to be created using floret inoculation techniques. Thus, the micro-slit technique (Latch and Christensen, 1985) remains the most efficient method for the creation of artificial associations. However, the low success rate of this technique has been an issue since its conception 22 years ago. Close to the completion date of this thesis, the creation of artificial associations using the micro-slit technique was attempted several times. Although a controlled experiment was not conducted, it was found that germinating seeds on a 3% (w/v) agar medium +3% (w/v) sucrose, prior to inoculation, was found to greatly increase the survival rate of the seedlings post inoculation to the stage at which they were potted up. Time precluded the examination of these seedlings

for the establishment of successful infections but this preliminary result suggests that the micro-slit technique developed by Latch and Christensen (1985) may be able to be modified to increase its current low success rate.

Although the floret inoculation of a grass host with a *Neotyphodium* endophyte was not successful, investigation into the use of floret inoculation techniques on grass plants produced methods by which plant resistance to other fungal pathogens, such as *Claviceps purpurea* (ergot) and *Gloeotinia temulenta* (blind seed disease), could be examined. Although they are yet to be tested, these methods may prove to be valuable in forage and turf grass plant breeding programs.

In this thesis, *Neotyphodium*-specific primers were successfully designed to be specific to the β-tubulin gene of *N. coenophialum*, *N. lolii* and *N. uncinatum*. The NeoS-F2 – NeoS-R2 primer pair was able to detect endophyte DNA at a concentration of 0.005 ng μl^{-1} and showed it was likely to be capable of detecting one endophyte infected seed in 1000 non-infected seeds. This primer pair also enabled discrimination between *N. uncinatum* and *N. coenophialum*/*N. lolii* via the amplification of products with different lengths. The NeoS-F2 – NeoS-R2 pair also showed it was more practical for use in commercial plant breeding programs than *Neotyphodium*-specific primers designed in previous studies (Doss et al., 1998; Dombrowski et al., 2006) because checking and adjusting the concentration of DNA extracted from endophyte infected plant material was not necessary for efficient PCR detection.

However, it was not possible, using the NeoS-F2 – NeoS-R2 primer pair, to relate the amount of endophyte DNA in a seed lot to its known viable endophyte infection rate. It is unlikely that the efficiency of the PCR reaction or the specificity of the primers caused this result. Natural variation in the amount of endophyte DNA in a group of 50 seedlings or the error involved in the extraction of DNA and preparation of the RT-qPCR reactions were the likely causes. Although in theory RT-qPCR might seem like a good method for determining infection rate of micro-organisms *in planta*, it has not yet been shown to

work experimentally. Future advances in molecular technology may help in the practical development of such a method.

Nick Grbavac (Agriquality, Lincoln) has recently developed a new protocol for the determination of the viable *Neotyphodium* endophyte infection rate of forage grass cultivars. This test involves isolating the endophyte from germinating seeds directly onto a medium to determine whether each seed contains viable endophyte or not (pers. comm. Nick Grbavac, Agriquality). This protocol eliminates the grow out period involved with the histochemical staining or immunoblot tests and takes a maximum of two weeks to produce a result. It is also less labour intensive than the histochemical staining method and less costly than the immunoblot method. This makes it the most efficient commercial method for determining the viable *Neotyphodium* endophyte infection rate of a seed lot. Agriquality will fully commercialise this test in 2007.

The understanding of *Neotyphodium* endophytes has increased rapidly since the discovery 30 years ago that they were responsible for livestock disorders. The discovery or creation of novel endophyte strains and the establishment of stable associations between them and grass cultivars with desirable agronomic characteristics was the major goal when research into *Neotyphodium* endophytes began and remains so today. Because of their implications with stock health, insect and drought resistance, novel strains of *Neotyphodium* endophytes are, commercially, very valuable. Some of the early methods used in the study of *Neotyphodium* endophytes were not developed for use in the commercial environment. However, many of these methods are still being used today as if they were developed for commercial purposes. Results and observations published in this thesis suggest that many of these original methods for culturing *Neotyphodium* endophytes *in vitro*, inoculating *Neotyphodium* endophytes into grass hosts, and testing the viable *Neotyphodium* endophyte infection rate of seed lots, may be able to be improved or modified in ways to make them more efficient for use in the commercial and/or research environments. Improving these methods assists an end goal of discovering or creating new novel strains of *Neotyphodium* endophytes and establishing stable associations between them and desirable grass cultivars.

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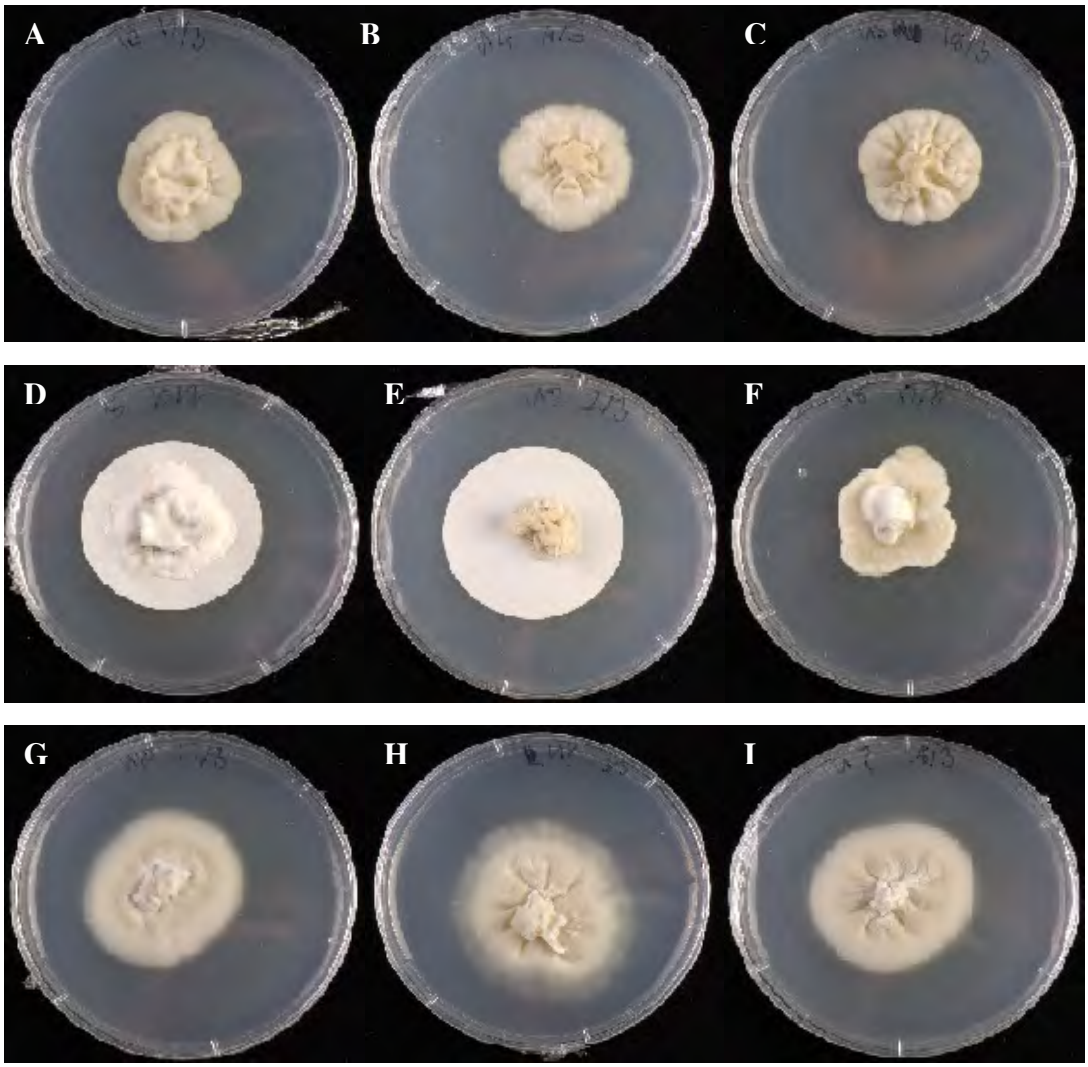
Appendix 1

The colony appearances of various strains of *Neotyphodium uncinatum*



Appendix 1. Various strains of *Neotyphodium uncinatum* isolated from *Festuca pratensis* (meadow fescue). (a) U3, (b) U4, (c) U5, (d) U6, (e) U7, (f) U8, (g) U10, (h) unnamed, and (i) unnamed strains of *N. uncinatum* cultured on 1.5% (w/v) agar malt extract agar. All strains have been shown to be of the *N. uncinatum* species by amplified fragment length polymorphism (AFLP) analysis (Cropmark Seeds Ltd., unpublished data)

Appendix 2

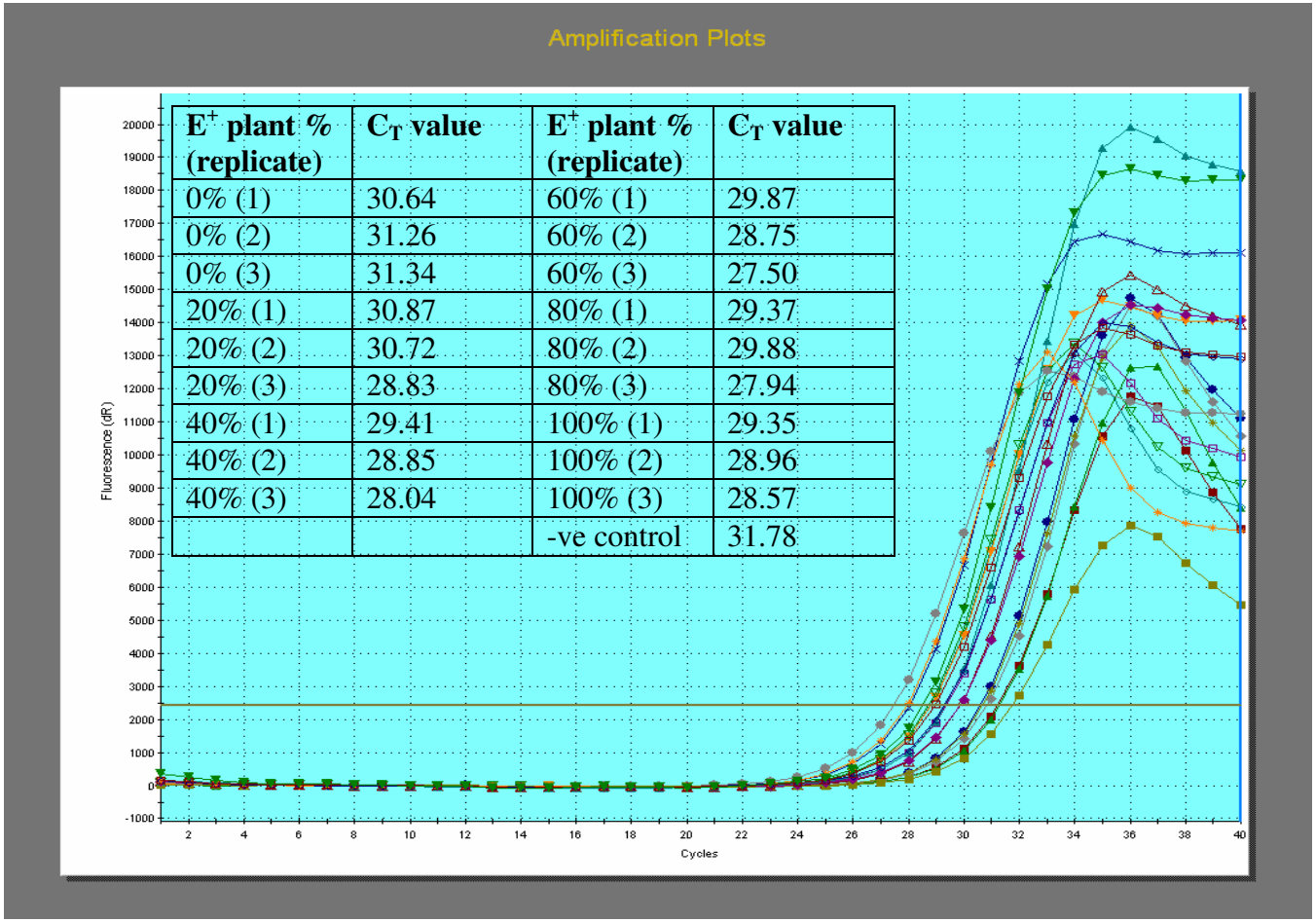
Composition of slow release potting mix used for growing all grass plants in this thesis

Appendix 2. Compositon of slow release potting mix used for growing all grass plants in this thesis

BULK INGREDIENTS	
Bark	80%
Bioblend (blood and bone)	20%
FERTILISER INGREDIENTS	**Kg per 2 m^3**
Dolomite	2.50
Gypsum Screened	3.00
Ag Lime	2.00
Enduro Short	0.70
Trace Element Mix	0.60
Nutri Start	2.00
Nutricote	3.00
Penetraide	3.00

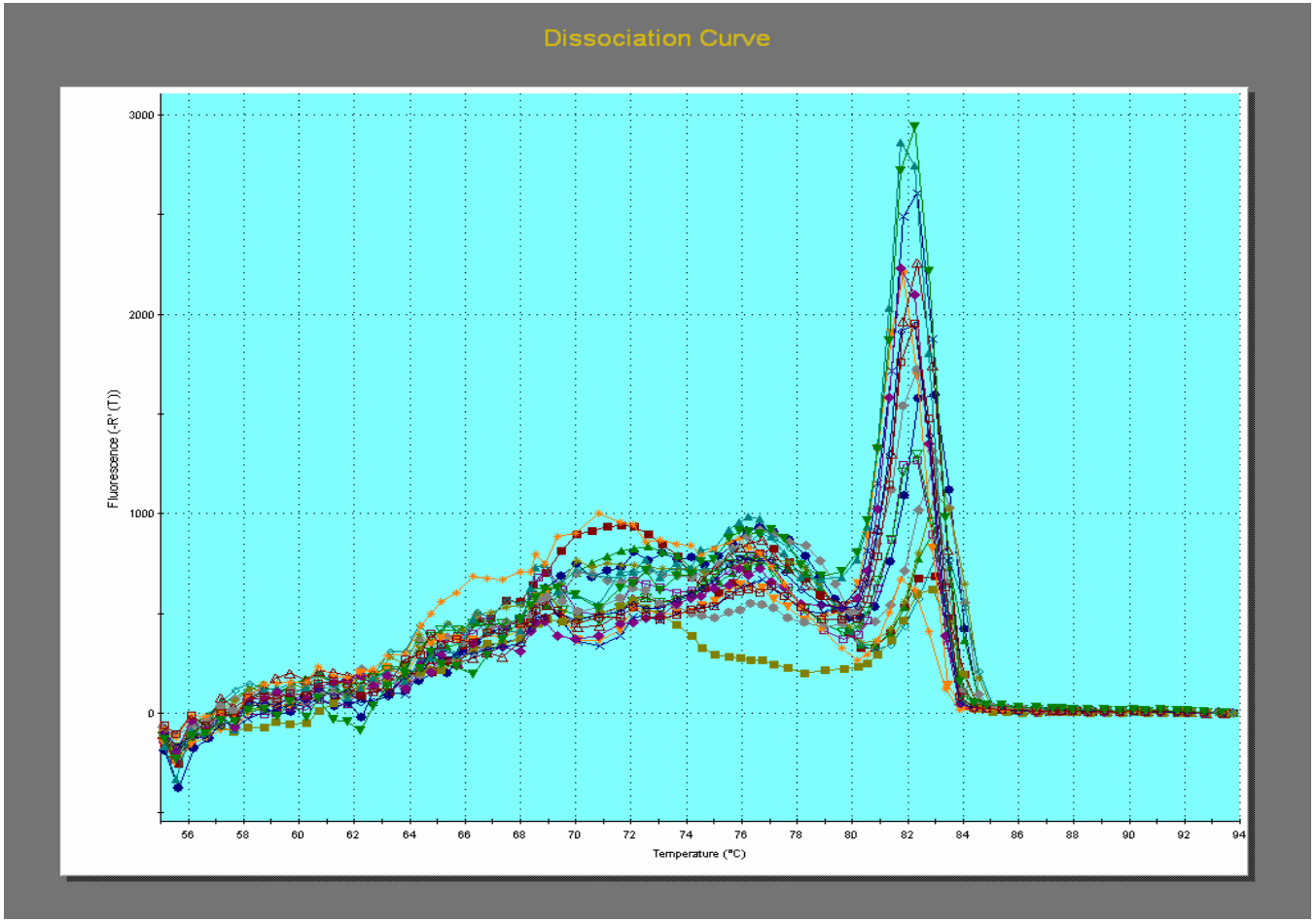
Appendix 3

Amplification plots and dissociation curves for protocol testing of FP889 (U2) and Revolution™ (AR1)

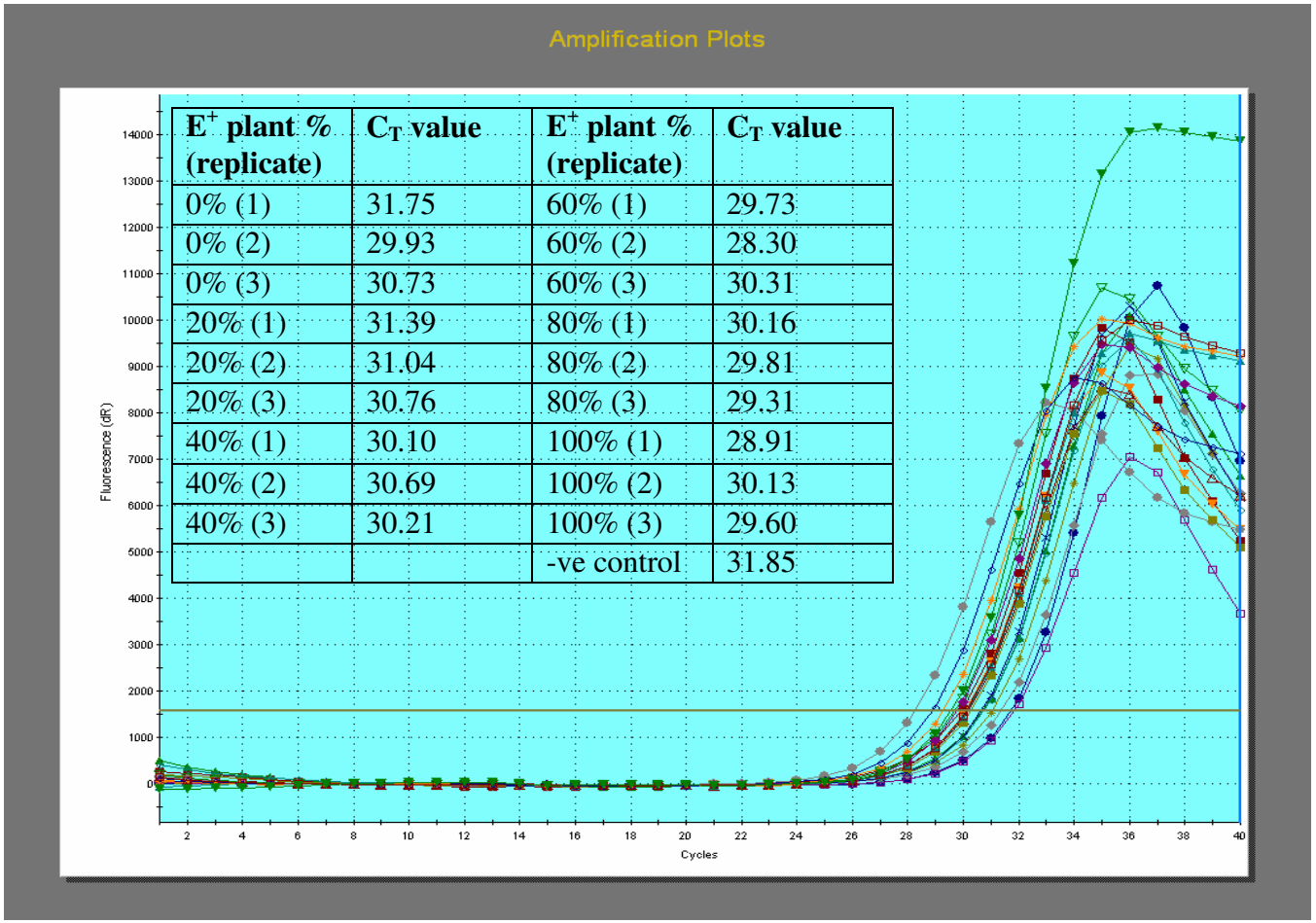


E^+ plant % (replicate)	C_T value	E^+ plant % (replicate)	C_T value
0% (1)	30.64	60% (1)	29.87
0% (2)	31.26	60% (2)	28.75
0% (3)	31.34	60% (3)	27.50
20% (1)	30.87	80% (1)	29.37
20% (2)	30.72	80% (2)	29.88
20% (3)	28.83	80% (3)	27.94
40% (1)	29.41	100% (1)	29.35
40% (2)	28.85	100% (2)	28.96
40% (3)	28.04	100% (3)	28.57
		-ve control	31.78

Appendix 3. Amplification plots and C_T values of the DNA extracted from the FP889 seedling replicates differing in their percentage of E^+ seedlings

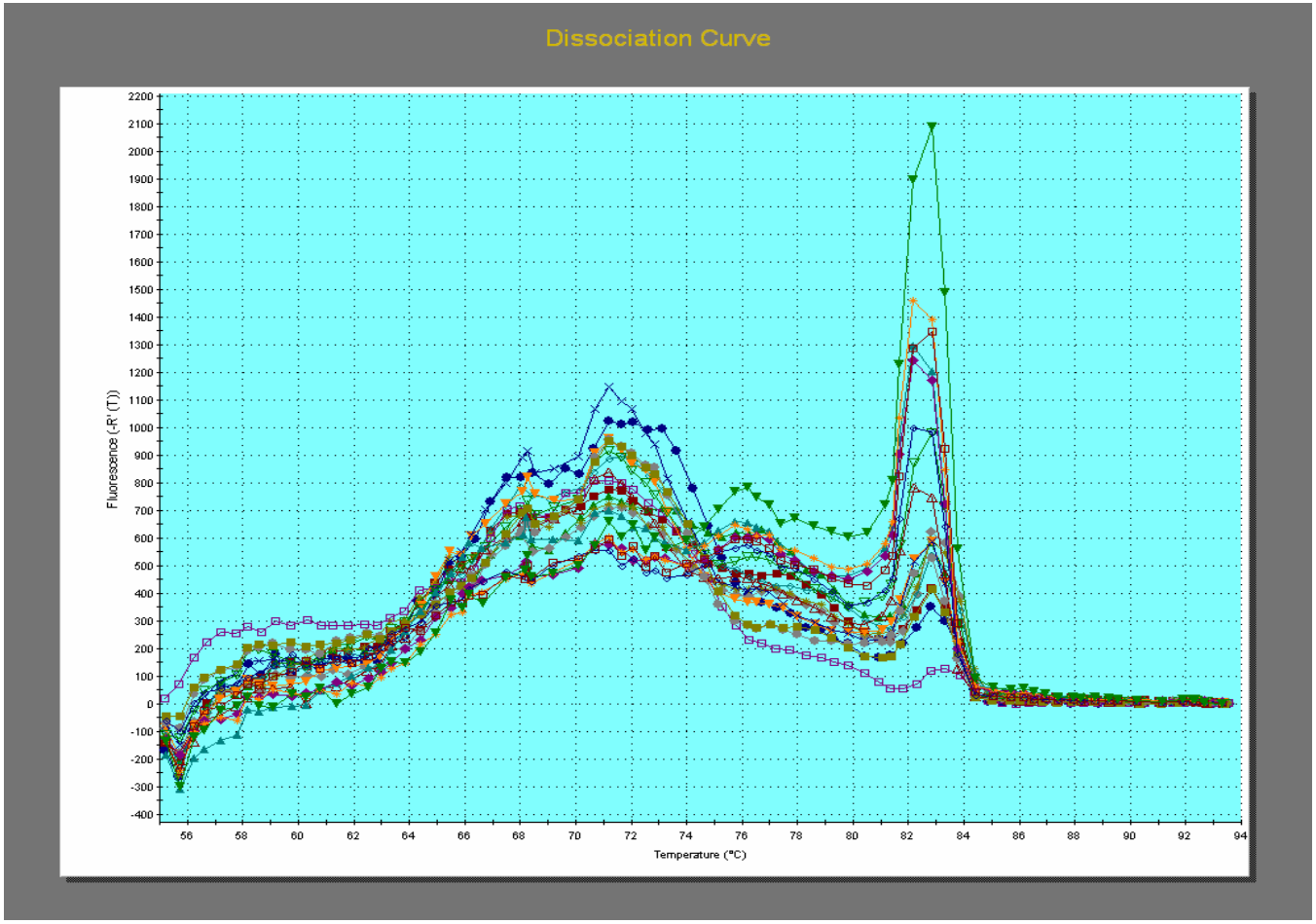


Appendix 3. Dissociation curves of the products generated by the NeoS-F2 – NeoS-R2 primer pair when tested on the DNA extracted from the FP889 seedling replicates differing in their percentage of E^+ seedlings



E^+ plant % (replicate)	C_T value	E^+ plant % (replicate)	C_T value
0% (1)	31.75	60% (1)	29.73
0% (2)	29.93	60% (2)	28.30
0% (3)	30.73	60% (3)	30.31
20% (1)	31.39	80% (1)	30.16
20% (2)	31.04	80% (2)	29.81
20% (3)	30.76	80% (3)	29.31
40% (1)	30.10	100% (1)	28.91
40% (2)	30.69	100% (2)	30.13
40% (3)	30.21	100% (3)	29.60
		-ve control	31.85

Appendix 3. Amplification plots and C_T values of the DNA extracted from the Revolution™ seedling replicates differing in their percentage of E^+ seedlings



Appendix 3. Dissociation curves of the products generated by the NeoS-F2 – NeoS-R2 primer pair when tested on the DNA extracted from the Revolution^{TM} seedling replicates differing in their percentage of E^{+} seedlings